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(54) Title: TUMOR HOMING MOLECULES, CONJUGATES DERIVED THEREFROM, AND METHODS OF USING SAME

(57) Abstract

The present invention provides tumor homing molecules, which selectively home to a tumor. The invention also provides methods of using a tumor homing molecule to target an agent such as a drug to a selected tumor or to identify the target molecule expressed by the tumor. The invention also provides methods of targeting a tumor containing angiogenic vasculature by contacting the tumor with a molecule that specifically binds an α_v-containing integrin. The invention further provides molecules that can selectively home to angiogenic vasculature. In addition, the invention provides a target molecule, which is specifically bound by a tumor homing molecule and is expressed by angiogenic vasculature. The invention also provides antibodies that bind to the target molecule and peptidomimetics that competitively inhibit binding of a ligand to the target molecule.

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TUMOR HOMING MOLECULES, CONJUGATES DERIVED THEREFROM, AND METHODS OF USING SAME

5   This application claims the benefit of priority
of U.S. Provisional Application No. 60/060,947, which was
converted from United States Serial No. 08/710,067, filed
September 10, 1996, the entire contents of which is
incorporated herein by reference.

15

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

20   The present invention relates generally to the
fields of cancer biology and drug delivery and, more
specifically, to peptides that selectively home to a
tumor, particularly a malignant tumor, to compositions
comprising an agent such as a therapeutic agent
25 conjugated to such tumor homing molecules, and to methods
of using such molecules to target an agent to a tumor.

BACKGROUND INFORMATION

30   Continuous developments over the past quarter
century have resulted in substantial improvements in the
ability of a physician to diagnose a cancer in a patient.
For example, antibody based assays such as that for
prostate specific antigen now allow early diagnosis of
35 cancers such as prostate cancer. More recently, methods
of genetic screening are becoming available to identify
persons that may be particularly susceptible to

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developing a cancer. Genetic screening methods are based
on the identification of one or more mutations in a gene
that correlates with the development of a cancer. For
example, the identification of genes such as BRCA1 and
5   BRCA2 allowed the further identification of mutations in
these genes that, in some cases, can correlate with
susceptibility to developing breast cancer.

Unfortunately, methods for treating cancer have
10 not kept pace with those for diagnosing the disease.
Thus, while the death rate from various cancers has
decreased due to the ability of a physician to detect the
disease at an earlier stage, the ability to treat
patients presenting with more advanced disease has
15 advanced only minimally.

A major hurdle to advances in treating cancer
is the relative lack of agents that can selectively
target the cancer, while sparing normal tissue. For
20 example, radiation therapy and surgery, which generally
are localized treatments, can cause substantial damage to
normal tissue in the treatment field, resulting in
scarring and, in severe cases, loss of function of the
normal tissue. Chemotherapy, in comparison, which
25 generally is administered systemically, can cause
substantial damage to organs such as bone marrow,
mucosae, skin and the small intestine, which undergo
rapid cell turnover and continuous cell division. As a
result, undesirable side effects such as nausea, loss of
30 hair and drop in blood cell count occur as a result of
systemically treating a cancer patient with
chemotherapeutic agents. Such undesirable side effects
often limit the amount of a treatment that can be
administered. Thus, cancer remains a leading cause of
35 patient morbidity and death.

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Efforts have been made to increase the target specificity of various drugs. For example, where a unique cell surface marker is expressed by a population of cells making up a tumor, an antibody can be raised
5   against the unique marker and a drug can be linked to the antibody. Upon administration of the drug/antibody complex to the patient, the binding of the antibody to the marker results in the delivery of a relatively high concentration of the drug to the tumor. Similar methods
10 can be used where a particular cancer cell or the supporting cell or matrix expresses a unique cell surface receptor or a ligand for a particular receptor. In these cases, the drug can be linked to the specific ligand or to the receptor, respectively, thus providing a means to
15 deliver a relatively high concentration of the drug to the tumor.

Tumors are characterized, in part, by a relatively high level of active angiogenesis, resulting
20 in the continual formation of new blood vessels to support the growing tumor. Such angiogenic blood vessels are distinguishable from mature vasculature. One of the distinguishing features of angiogenic vasculature is that unique endothelial cell surface markers are expressed.
25 Thus, the blood vessels in a tumor provide a potential target for directing a chemotherapeutic agent to the tumor, thereby reducing the likelihood that the agent will kill sensitive normal tissues. Furthermore, if agents that target the angiogenic blood vessels in a
30 tumor can be identified, there is a likelihood that the agents can be useful against a variety of different types of tumors, since it is the target molecules in the angiogenic vessels that are recognized by such agents and not receptors specific for the tumor cells. However, the
35 use of molecules that can bind specifically to tumor vasculature and target a chemotherapeutic agent to the tumor has not been demonstrated.

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While linking a drug to a molecule that homes
to a tumor can provide significant advantages for
treatment over the use of a drug, alone, use of this
method is severely limited by the scarcity of useful cell
5   surface markers expressed in a tumor. Thus, a need
exists to identify molecules that can selectively home to
a tumor, particularly to the vasculature supporting the
tumor. The present invention satisfies this need and
provides related advantages as well.
10

SUMMARY OF THE INVENTION

The present invention relates to molecules that
selectively home to tumors, generally to the vasculature
15   supporting the tumor. For example, the invention
provides tumor homing peptides that contain, for example,
the motif asparagine-glycine-arginine (NGR) or glycine-
serine-leucine (GSL), or the α_v-containing integrin
binding motif, arginine-glycine-aspartic acid (RGD).
20

The invention also relates to compositions
comprising a tumor homing molecule, such as a tumor
homing peptide, linked to a moiety to produce a tumor
homing molecule/moiety conjugate. Such a moiety can be a
25   drug, for example, a cancer therapeutic agent such as
doxorubicin, taxol, cis-platinum, or the like, in which
case the tumor homing molecule/moiety conjugate provides
a therapeutic reagent. A moiety conjugated to a tumor
homing molecule also can be a detectable label, for
30   example, a radionuclide or paramagnetic spin label, such
that the molecule/moiety conjugate provides a diagnostic
reagent.

The invention also relates to methods of
35   targeting a moiety such as a drug to a tumor by
contacting the tumor homing molecule/moiety conjugate
with the tumor. Thus, the invention provides methods of

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diagnosing or treating a cancer in a subject by
administering a composition comprising a tumor homing
molecule conjugated to a cancer therapeutic agent to the
subject.   For example, administration of a composition
5   comprising a doxorubicin/CDCRGDCFC (SEQ ID NO: 1)
conjugate to a mouse bearing a transplanted breast
carcinoma substantially reduced the growth of the breast
cancer and the number of metastases and resulted in
substantially greater survival as compared to tumor
10   bearing mice treated with doxorubicin, alone, or with
doxorubicin conjugated to an unrelated peptide.

The invention further relates to methods of
identifying a target molecule in a tumor by detecting
15   selective binding of the target molecule to a tumor
homing molecule.   For example, a peptide that selectively
homes to a tumor can be attached to a solid matrix for
use in affinity chromatography.   A sample of the tumor
can be obtained and passed over the affinity matrix under
20   conditions that allow specific binding of the target
molecule, which then can be collected and identified
using well known biochemical methods.   Thus, the
invention also provides a target molecule, which acts as
a receptor for a tumor homing molecule.   Such a target
25   molecule can be useful, for example, for raising an
antibody specific for the target molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

30   Figures 1A to 1V show the immunohistochemical
staining of phage expressing the NGR tumor homing
peptide, CNGRCVSGCAGRC (SEQ ID NO: 3; "NGR phage"), in
tumors and normal tissues following intravenous injection
into nude mice bearing a human breast carcinoma or a
35   human Kaposi's sarcoma.   Samples were taken 4 min
(Figures 1E, 1G, 1H and 1J) or 24 hr (Figures 1A to 1D,
1F, 1I, and 1K to 1V) after administration of the phage.

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Figures 1A, 1C, 1G and 1J are from mice receiving
insertless phage (control phage) and Figures 1B, 1D, 1E,
1F, 1H, 1I and 1K to 1V are from mice receiving NGR
phage. Figures 1A, 1B, 1E, 1F and 1G are breast tumor
5 samples; Figures 1C, 1D, 1H, 1I and 1J are Kaposi's
sarcoma samples; Figure 1K is brain; Figure 1L is lymph
node; Figure 1M is kidney; Figure 1N is pancreas;
Figure 1O is uterus; Figure 1P is mammary fat pad;
Figure 1Q is lung; Figure 1R is intestine; Figure 1S is
10 skin; Figure 1T is skeletal muscle; Figure 1U is heart
and Figure 1V is urinary tract epithelium.
Magnification: Figures 1A to 1D, 40x; Figures 1E to 1V,
200x.

15   Figures 2A to 2F show the hematoxylin and eosin
stained tumor samples from representative pairs of mice
treated two times with 5 μg equivalent of doxorubicin
(Figures 2A to 2C) or doxorubicin/CDCRGDCFC (SEQ ID
NO: 1) conjugate (Figures 2D to 2F). Paired mice had
20 size matched tumors at the time treatment was initiated.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to molecules that
25 selectively home to tumors. For example, the invention
provides tumor homing peptides such as the peptides
CGRECPRLCQSSC (SEQ ID NO: 2) and CNGRCVSGCAGRC (SEQ ID
NO: 3), which were identified based on their ability to
home to a breast carcinoma, and the peptide CLSGSLSC (SEQ
30 ID NO: 4, which was identified based on its ability to
home to a melanoma. Such tumor homing peptides were
identified using *in vivo* panning (see United States
Patent No. 5,622,699, issued April 22, 1997; Pasqualini
and Ruoslahti, Nature 380:364-366 (1996), each of which
35 is incorporated herein by reference).

The disclosed tumor homing peptides were identified based on their homing to various particular tumors. For example, *in vivo* panning was performed using a mouse bearing a human breast carcinoma xenograft and

5   peptides that homed to the breast tumor were identified. However, as disclosed herein, such tumor homing peptides generally homed to other types of tumors, including a mouse melanoma and a human Kaposi's sarcoma. Thus, while the tumor homing peptide CNGRCVSGCAGRC (SEQ ID NO: 3) was

10 identified by its ability to home *in vivo* to a breast tumor, this peptide also homed *in vivo* to a melanoma and to a Kaposi's sarcoma, but not to nontumor tissues.

Similarly, the tumor homing peptide CLSGSLSC

15 (SEQ ID NO: 4) was identified based on its homing to melanoma. However, further examination of this peptide revealed that it also homed to a breast tumor and to Kaposi's sarcoma. Immunohistological analysis revealed that such tumor homing peptides initially are associated

20 with the vasculature of the various tumors, although at later time the molecules are associated with tumor parenchymal cells. Thus, the general tumor homing ability of a tumor homing molecule of the invention is due, at least in part, to the ability of the tumor homing

25 molecules to target angiogenic vasculature associated with a tumor. These results indicate that specific target molecules are expressed by the cells comprising the vasculature in a tumor as compared to the cell surface molecule expressed by vasculature in nontumor

30 tissues. Using methods as disclosed herein, the artisan readily can determine whether a tumor homing molecule homes generally to the angiogenic vasculature associated with a tumor or homes specifically to a particular type of tumor cell.

35

As used herein, the term "tumor" means a mass of cells that are characterized, at least in part, by

containing angiogenic vasculature. The term "tumor" is
used broadly to include the tumor parenchymal cells as
well as the supporting stroma, including the angiogenic
blood vessels that infiltrate the tumor parenchymal cell
5   mass. Although a tumor generally is a malignant tumor,
i.e., a "cancer," a tumor also can be nonmalignant,
provided that neovascularization is associated with the
tumor. The term "normal" or "nontumor" tissue is used to
refer to tissue that is not a "tumor." As disclosed
10 herein, a tumor homing molecule can be identified based
on its ability to home a tumor, but not to a
corresponding nontumor tissue.

        As used herein, the term "corresponding," when
15 used in reference to tumors or tissues or both, means
that two or more tumors, or two or more tissues, or a
tumor and a tissue are of the same histologic type. The
skilled artisan will recognize that the histologic type
of a tissue is a function of the cells comprising the
20 tissue. Thus, the artisan will recognize, for example,
that a nontumor tissue corresponding to a breast tumor is
normal breast tissue, whereas a nontumor tissue
corresponding to a melanoma is skin, which contains
melanocytes. Furthermore, for purposes of the invention,
25 it is recognized that a tumor homing molecule can bind
specifically to a target molecule expressed by the
vasculature in a tumor, which generally contains blood
vessels undergoing neovascularization, in which case a
tissue corresponding to the tumor would comprise nontumor
30 tissue containing blood vessels that are not undergoing
active angiogenesis.

        The term "corresponding" also is used herein in
reference to the evolutionarily conserved nature of
35 target molecules, which are expressed in a tumor, for
example, in a mouse as compared to a human. Thus,
reference to the corresponding target molecules in mouse

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tumor vasculature as compared, for example, to human
vasculature, means target molecules having a similar
function, particularly the ability to specifically bind a
tumor homing molecule.

5

Identified tumor homing molecules are useful,
for example, for targeting a desired moiety such as a
drug, a toxin or a detectable label, which can be linked
to the molecule, to a tumor. Thus, the invention
10   provides tumor homing molecule/moiety conjugates, which
are useful for targeting the moiety to a tumor.
Accordingly, the invention also provides methods of
targeting a moiety to a tumor and, therefore, methods of
reducing the severity of a tumor and of treating a
15   subject having a cancer (see Example VII). In addition,
a tumor homing molecule is useful for identifying the
target molecule, to which the homing molecule binds in
the tumor.

20   A tumor homing molecule can be identified by
screening a library of molecules by *in vivo* panning as
disclosed herein. As used herein, the term "library"
means a collection of molecules. A library can contain a
few or a large number of different molecules, varying
25   from about ten molecules to several billion molecules or
more. If desired, a molecule can be linked to a tag,
which can facilitate recovery or identification of the
molecule.

30   As used herein, the term "molecule" is used
broadly to mean an organic chemical such as a drug; a
nucleic acid molecule such as an RNA, a cDNA or an
oligonucleotide; a peptide, including a variant or
modified peptide or peptide-like molecules, referred to
35   herein as peptidomimetics, which mimic the activity of a
peptide; or a protein such as an antibody or a growth
factor receptor or a fragment thereof such as an Fv, Fd

10

or Fab fragment of an antibody, which contains a binding
domain. For convenience, the term "peptide" is used
broadly herein to mean peptides, proteins, fragments of
proteins and the like. A molecule also can be a
5   non-naturally occurring molecule, which does not occur in
nature, but is produced as a result of *in vitro* methods,
or can be a naturally occurring molecule such as a
protein or fragment thereof expressed from a cDNA library
or a peptidomimetic.
10

As used herein, the term "peptidomimetic" is
used broadly to mean a peptide-like molecule that has the
binding activity of the tumor homing peptide. With
respect to the tumor homing peptides of the invention,
15   peptidomimetics, which include chemically modified
peptides, peptide-like molecules containing non-naturally
occurring amino acids, peptoids and the like, have the
binding activity of a tumor homing peptide upon which the
peptidomimetic is derived (see, for example, "Burger's
20   Medicinal Chemistry and Drug Discovery" 5th ed., vols. 1
to 3 (ed. M.E. Wolff; Wiley Interscience 1995), which is
incorporated herein by reference). Peptidomimetics
provide various advantages over a peptide, including that
a peptidomimetic can be stable during passage through the
25   digestive tract and, therefore, useful for oral
administration.

Methods for identifying a peptidomimetic are
well known in the art and include, for example, the
30   screening of databases that contain libraries of
potential peptidomimetics. For example, the Cambridge
Structural Database contains a collection of greater than
300,000 compounds that have known crystal structures
(Allen et al., Acta Crystallogr. Section B, 35:2331
35   (1979)). This structural depository is continually
updated as new crystal structures are determined and can
be screened for compounds having suitable shapes, for

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example, the same shape as a tumor homing molecule, as
well as potential geometrical and chemical
complementarity to a target molecule bound by a tumor
homing peptide. Where no crystal structure of a tumor
5   homing peptide or a target molecule, which binds the
tumor homing molecule, is available, a structure can be
generated using, for example, the program CONCORD
(Rusinko et al., J. Chem. Inf. Comput. Sci. 29:251
(1989)). Another database, the Available Chemicals
10 Directory (Molecular Design Limited, Informations
Systems; San Leandro CA), contains about 100,000
compounds that are commercially available and also can be
searched to identify potential peptidomimetics of a tumor
homing molecule.
15

        Methods for preparing libraries containing
diverse populations of various types of molecules such as
peptides, peptoids and peptidomimetics are well known in
the art and various libraries are commercially available
20 (see, for example, Ecker and Crooke, Biotechnology
13:351-360 (1995), and Blondelle et al., Trends Anal.
Chem. 14:83-92 (1995), and the references cited therein,
each of which is incorporated herein by reference; see,
also, Goodman and Ro, Peptidomimetics for Drug Design, in
25 "Burger's Medicinal Chemistry and Drug Discovery" Vol. 1
(ed. M.E. Wolff; John Wiley & Sons 1995), pages 803-861,
and Gordon et al., J. Med. Chem. 37:1385-1401 (1994),
each of which is incorporated herein by reference).
Where a molecule is a peptide, protein or fragment
30 thereof, the molecule can be produced *in vitro* directly
or can be expressed from a nucleic acid, which can be
produced *in vitro*. Methods of synthetic peptide and
nucleic acid chemistry are well known in the art.

35   A library of molecules also can be produced,
for example, by constructing a cDNA expression library
from mRNA collected from a cell, tissue, organ or

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organism of interest. Methods for producing such
libraries are well known in the art (see, for example,
Sambrook et al., Molecular Cloning: A laboratory manual
(Cold Spring Harbor Laboratory Press 1989), which is
5   incorporated herein by reference). Preferably, a peptide
encoded by the cDNA is expressed on the surface of a cell
or a virus containing the cDNA. For example, cDNA can be
cloned into a phage vector such as fuse 5 (Example I),
wherein, upon expression, the encoded peptide is
10   expressed as a fusion protein on the surface of the
phage.

In addition, a library of molecules can
comprise a library of nucleic acid molecules, which can
15   be DNA or RNA or an analog thereof. Nucleic acid
molecules that bind, for example, to a cell surface
receptor are well known (see, for example, O'Connell et
al., Proc. Natl. Acad. Sci., USA 93:5883-5887 (1996);
Tuerk and Gold, Science 249:505-510 (1990); Gold et al.,
20   Ann. Rev. Biochem. 64:763-797 (1995), each of which is
incorporated herein by reference). Thus, a library of
nucleic acid molecules can be administered to a subject
having a tumor and tumor homing molecules can be
identified by *in vivo* panning. If desired, the nucleic
25   acid molecules can be nucleic acid analogs that, for
example, are less susceptible to attack by nucleases
(see, for example, Jelinek et al., Biochemistry 34:11363-
11372 (1995); Latham et al., Nucl. Acids Res. 22:2817-
2822 (1994); Tam et al., Nucl. Acids Res. 22:977-986
30   (1994); Reed et al., Cancer Res. 59:6565-6570 (1990),
each of which is incorporated herein by reference).

As disclosed herein, *in vivo* panning for the
purpose of identifying a tumor homing molecule comprises
35   administering a library to a subject, collecting a sample
of a tumor and identifying a tumor homing molecule. The
presence of a tumor homing molecule can be identified

●   ●

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using various methods well known in the art. Generally,
the presence of a tumor homing molecule in a tumor is
identified based on one or more characteristics common to
the molecules present in the library, then the structure
of a particular tumor homing molecule is identified. For
example, a highly sensitive detection method such as mass
spectrometry, either alone or in combination with a
method such as gas chromatography, can be used to
identify tumor homing molecules in a tumor. Thus, where
a library comprises diverse molecules based generally on
the structure of an organic molecule such as a drug, a
tumor homing molecule can be identified by determining
the presence of a parent peak for the particular
molecule.

If desired, the tumor can be collected, then
processed using a method such as HPLC, which can provide
a fraction enriched in molecules having a defined range
of molecular weights or polar or nonpolar characteristics
or the like, depending, for example, on the general
characteristics of the molecules comprising the library.
Conditions for HPLC will depend on the chemistry of the
particular molecule and are well known to those skilled
in the art. Similarly, methods for bulk removal of
potentially interfering cellular materials such as DNA,
RNA, proteins, lipids or carbohydrates are well known in
the art, as are methods for enriching a fraction
containing an organic molecule using, for example,
methods of selective extraction. Where a library
comprises a population of diverse organic chemical
molecules, each linked to a specific oligonucleotide tag,
such that the specific molecule can be identified by
determining the oligonucleotide sequence using polymerase
chain reaction (PCR), genomic DNA can be removed from the
sample of the collected tumor in order to reduce the
potential for background PCR reactions. In addition, a
library can comprise a population of diverse molecules

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such as organic chemical molecules, each linked to a common, shared tag. Based on the presence and properties of the shared tag, molecules of the library that selectively home to a tumor can be substantially isolated

5   from a sample of the tumor. These and other methods can be useful for enriching a sample of a collected tumor for the particular tumor homing molecule, thereby removing potentially contaminating materials from the collected tumor sample and increasing the sensitivity of detecting

10   a molecule.

       Evidence provided herein indicates that a sufficient number of tumor homing molecules selectively homes to a tumor during *in vivo* panning such that the

15   molecules readily can be identified. For example, various independent phage expressing the same peptide were identified in tumors formed from implanted human breast cancer cells (Table 1), from mouse melanoma cells (Table 2) or from human Kaposi's sarcoma cells (Table 3).

20

       Although a substantial fraction of the identified tumor homing molecules have the same structure, the peptide inserts of only a small number of isolated phage were determined. It should be recognized,

25   however, that hundreds of thousands to millions of phage expressing organ homing peptides have been recovered following *in vivo* pannings for organ homing molecules (see, for example, U.S. Patent No. 5,622,699; Pasqualini and Ruoslahti, *supra*, 1996). These results indicate that

30   specific tumor homing molecules will be present in substantial numbers in a tumor following *in vivo* homing, thereby increasing the ease with which the molecules can be identified.

35   Ease of identification of a tumor homing molecule, particularly an untagged molecule, depends on various factors, including the presence of potentially

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contaminating background cellular material. Thus, where
the tumor homing molecule is an untagged peptide, a
larger number must home to the tumor in order to identify
the specific peptides against the background of cellular

5   protein. In contrast, a much smaller number of an
untagged organic chemical homing molecule such as a drug
is identifiable because such molecules normally are
absent from or present in only small numbers in the body.
In such a case, a highly sensitive method such as mass

10   spectrometry can be used to identify a tumor homing
molecule. The skilled artisan will recognize that the
method of identifying a molecule will depend, in part, on
the chemistry of the particular molecule.

15   Where a tumor homing molecule is a nucleic acid
or is tagged with a nucleic acid, an assay such as PCR
can be particularly useful for identifying the presence
of the molecule because, in principle, PCR can detect the
presence of a single nucleic acid molecule (see, for

20   example, Erlich, PCR Technology: Principles and
Applications for DNA Amplification (Stockton Press 1989),
which is incorporated herein by reference). Preliminary
studies have demonstrated that, following intravenous
injection of 10 ng of an approximately 6000 base pair

25   plasmid into a mouse and 2 minutes in the circulation,
the plasmid was detectable by PCR in a sample of lung.
These results indicate that nucleic acids are
sufficiently stable when administered into the
circulation such that in vivo panning can be used to

30   identify nucleic acid molecules that selectively home to
a tumor.

     The molecules of a library can be tagged, which
can facilitate recovery or identification of the

35   molecule. As used herein, the term "tag" means a
physical, chemical or biological moiety such as a plastic
microbead, an oligonucleotide or a bacteriophage,

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respectively, that is linked to a molecule of the
library. Methods for tagging a molecule are well known
in the art (Hermanson, _Bioconjugate Techniques_ (Academic
Press 1996), which is incorporated herein by reference).
5.

        A tag, which can be a shared tag or a specific
tag, can be useful for identifying the presence or
structure of a tumor homing molecule of a library. As
used herein, the term "shared tag" means a physical,
10   chemical or biological moiety that is common to each
molecule in a library. Biotin, for example, can be a
shared tag that is linked to each molecule in a library.
A shared tag can be useful to identify the presence of a
molecule of the library in a sample and also can be
15   useful to substantially isolate the molecules from a
sample. For example, where the shared tag is biotin, the
biotin-tagged molecules in a library can be substantially
isolated by binding to streptavidin or their presence can
be identified by binding with a labeled streptavidin.
20   Where a library is a phage display library, the phage
that express the peptides are another example of a shared
tag, since each peptide of the library is linked to a
phage. In addition, a peptide such as the hemaglutinin
antigen can be a shared tag that is linked to each
25   molecule in a library, thereby allowing the use of an
antibody specific for the hemaglutinin antigen to
substantially isolate molecules of the library from a
sample of a selected tumor.

30   A shared tag also can be a nucleic acid
sequence that can be useful to identify the presence of
molecules of the library in a sample or to substantially
isolate molecules of a library from a sample. For
example, each of the molecules of a library can be linked
35   to the same selected nucleotide sequence, which
constitutes the shared tag. An affinity column
containing a nucleotide sequence that is complementary to

the shared tag then can be used to hybridize molecules of
the library containing the shared tag, thus substantially
isolating the molecules from a tumor sample. A
nucleotide sequence complementary to a portion of the

5   shared nucleotide sequence tag also can be used as a PCR
primer such that the presence of molecules containing the
shared tag can be identified in a sample by PCR.

            A tag also can be a specific tag. As used

10   herein, the term "specific tag" means a physical,
chemical or biological tag that is linked to a particular
molecule in a library and is unique for that particular
molecule. A specific tag is particularly useful if it is
readily identifiable. A nucleotide sequence that is

15   unique for a particular molecule of a library is an
example of a specific tag. For example, the method of
synthesizing peptides tagged with a unique nucleotide
sequence provides a library of molecules, each containing
a specific tag, such that upon determining the nucleotide

20   sequence, the identity of the peptide is known (see
Brenner and Lerner, *Proc. Natl. Acad. Sci., USA* 89:5381-
5383 (1992), which is incorporated herein by reference).
The use of a nucleotide sequence as a specific tag for a
peptide or other type of molecule provides a simple means

25   to identify the presence of the molecule in a sample
because an extremely sensitive method such as PCR can be
used to determine the nucleotide sequence of the specific
tag, thereby identifying the sequence of the molecule
linked thereto. Similarly, the nucleic acid sequence

30   encoding a peptide expressed on a phage is another
example of a specific tag, since sequencing of the
specific tag identifies the amino acid sequence of the
expressed peptide.

35   The presence of a shared tag or a specific tag
can provide a means to identify or recover a tumor homing
molecule of the invention following *in vivo* panning. In

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addition, the combination of a shared tag and specific tag can be particularly useful for identifying a tumor homing molecule. For example, a library of peptides can be prepared such that each is linked to a specific
5   nucleotide sequence tag (see, for example, Brenner and Lerner, *supra*, 1992), wherein each specific nucleotide sequence tag has incorporated therein a shared tag such as biotin. Upon homing to a tumor, the particular tumor homing peptides can be substantially isolated from a
10   sample of the tumor based on the shared tag and the specific peptides can be identified, for example, by PCR of the specific tag (see Erlich, *supra*, 1989).

A tag also can serve as a support. As used
15   herein, the term "support" means a tag having a defined surface to which a molecule can be attached. In general, a tag useful as a support is a shared tag. For example, a support can be a biological tag such as a virus or virus-like particle such as a bacteriophage ("phage"); a
20   bacterium such as *E. coli*; or a eukaryotic cell such as a yeast, insect or mammalian cell; or can be a physical tag such as a liposome or a microbead, which can be composed of a plastic, agarose, gelatin or other biological or inert material. If desired, a shared tag useful as a
25   support can have linked thereto a specific tag. Thus, the phage display libraries used in the exemplified methods can be considered to consist of the phage, which is a shared tag that also is a support, and the nucleic acid sequence encoding the expressed peptide, the nucleic
30   acid sequence being a specific tag.

In general, a support should have a diameter less than about 10 μm to about 50 μm in its shortest dimension, such that the support can pass relatively
35   unhindered through the capillary beds present in the subject and not occlude circulation. In addition, a support can be nontoxic, so that it does not perturb the

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normal expression of cell surface molecules or normal
physiology of the subject, and biodegradable,
particularly where the subject used for *in vivo* panning
is not sacrificed to collect a selected tumor.

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Where a molecule is linked to a support, the
tagged molecule comprises the molecule attached to the
surface of the support, such that the part of the
molecule suspected of being able to interact with a
10 target molecule in a cell in the subject is positioned so
as to be able to participate in the interaction. For
example, where the tumor homing molecule is suspected of
being a ligand for a growth factor receptor, the binding
portion of the molecule attached to a support is
15 positioned so it can interact with the growth factor
receptor on a cell in the tumor. If desired, an
appropriate spacer molecule can be positioned between the
molecule and the support such that the ability of the
potential tumor homing molecule to interact with the
20 target molecule is not hindered. A spacer molecule also
can contain a reactive group, which provides a convenient
and efficient means of linking a molecule to a support
and, if desired, can contain a tag, which can facilitate
recovery or identification of the molecule (see
25 Hermanson, *supra*, 1996).

As exemplified herein, a peptide suspected of
being able to home to a selected tumor such as a breast
carcinoma or a melanoma was expressed as the N-terminus
30 of a fusion protein, wherein the C-terminus consisted of
a phage coat protein. Upon expression of the fusion
protein, the C-terminal coat protein linked the fusion
protein to the surface of a phage such that the
N-terminal peptide was in a position to interact with a
35 target molecule in the tumor. Thus, a molecule having a
shared tag was formed by the linking of a peptide to a
phage, wherein the phage provided a biological support,

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the peptide molecule was linked as a fusion protein, the
phage-encoded portion of the fusion protein acted as a
spacer molecule, and the nucleic acid encoding the
peptide provided a specific tag allowing identification
5   of a tumor homing peptide.

        As used herein, the term "in vivo panning,"
when used in reference to the identification of a tumor
homing molecule, means a method of screening a library by
10 administering the library to a subject and identifying a
molecule that selectively homes to a tumor in the subject
(see U.S. Patent No. 5,622,699). The term "administering
to a subject", when used in referring to a library of
molecules or a portion of such a library, is used in its
15 broadest sense to mean that the library is delivered to a
tumor in the subject, which, generally, is a vertebrate,
particularly a mammal such as a human.

        A library can be administered to a subject, for
20 example, by injecting the library into the circulation of
the subject such that the molecules pass through the
tumor; after an appropriate period of time, circulation
is terminated by sacrificing the subject or by removing a
sample of the tumor (Example I; see, also, U.S. Patent
25 No. 5,622,699; Pasqualini and Ruoslahti, supra, 1996).
Alternatively, a cannula can be inserted into a blood
vessel in the subject, such that the library is
administered by perfusion for an appropriate period of
time, after which the library can be removed from the
30 circulation through the cannula or the subject can be
sacrificed to collect the tumor, or the tumor can be
sampled, to terminate circulation. Similarly, a library
can be shunted through one or a few organs, including the
tumor, by cannulation of the appropriate blood vessels in
35 the subject. It is recognized that a library also can be
administered to an isolated perfused tumor. Such panning
in an isolated perfused tumor can be useful to identify

molecules that bind to the tumor and, if desired, can be
used as an initial screening of a library.

The use of *in vivo* panning to identify tumor
homing molecules is exemplified herein by screening a
phage peptide display library in tumor-bearing mice and
identifying specific peptides that selectively homed to a
breast tumor or to a melanoma tumor (Example I).
However, phage libraries that display protein receptor
molecules, including, for example, an antibody or an
antigen binding fragment of an antibody such an Fv, Fd or
Fab fragment; a hormone receptor such as a growth factor
receptor; or a cell adhesion receptor such as an integrin
or a selectin also can be used to practice the invention.
Variants of such molecules can be constructed using well
known methods such as random, site-directed or codon
based mutagenesis (see Huse, U.S. Patent No. 5,264,563,
issued November 23, 1993, which is incorporated herein by
reference) and, if desired, peptides can be chemically
modified following expression of the phage but prior to
administration to the subject. Thus, various types of
phage display libraries can be screened by *in vivo*
panning.

Phage display technology provides a means for
expressing a diverse population of random or selectively
randomized peptides. Various methods of phage display
and methods for producing diverse populations of peptides
are well known in the art. For example, Ladner et al.
(U.S. Patent No. 5,223,409, issued June 29, 1993, which
is incorporated herein by reference) describe methods for
preparing diverse populations of binding domains on the
surface of a phage. In particular, Ladner et al.
describe phage vectors useful for producing a phage
display library, as well as methods for selecting
potential binding domains and producing randomly or
selectively mutated binding domains.

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Similarly, Smith and Scott (Meth. Enzymol. 217:228-257 (1993); see, also, Scott and Smith, Science 249: 386-390 (1990), each of which is incorporated herein by reference) describe methods of producing phage peptide
5   display libraries, including vectors and methods of diversifying the population of peptides that are expressed (see, also, Huse, WO 91/07141 and WO 91/07149, each of which is incorporated herein by reference; see, also, Example I). Phage display technology can be
10   particularly powerful when used, for example, with a codon based mutagenesis method, which can be used to produce random peptides or randomly or desirably biased peptides (Huse, U.S. Patent No. 5,264,563, supra, 1993). These or other well known methods can be used to produce
15   a phage display library, which can be subjected to the in vivo panning method of the invention in order to identify a peptide that homes to a tumor.

In addition to screening a phage display
20   library, in vivo panning can be used to screen various other types of libraries, including, for example, an RNA or DNA library or a chemical library. If desired, the tumor homing molecule can be tagged, which can facilitate recovery of the molecule from the tumor or identification
25   of the molecule in the tumor. For example, where a library of organic molecules, each containing a shared tag, is screened, the tag can be a moiety such as biotin, which can be linked directly to the molecule or can be linked to a support containing the molecules. Biotin
30   provides a shared tag useful for recovering the molecule from a selected tumor sample using an avidin or streptavidin affinity matrix. In addition, a molecule or a support containing a molecule can be linked to a hapten such as 4-ethoxy-methylene-2-phenyl-2-oxazoline-5-one
35   (phOx), which can be bound by an anti-phOx antibody linked to a magnetic bead as a means to recover the molecule. Methods for purifying biotin or phOx labeled

conjugates are known in the art and the materials for
performing these procedures are commercially available
(e.g., Invitrogen, La Jolla CA; and Promega Corp.,
Madison WI). In the case where a phage library is
5   screened, the phage can be recovered using methods as
disclosed in Example I.

In vivo panning provides a method for directly
identifying molecules that can selectively home to a
10   tumor. As used herein, the term "home" or "selectively
home" means that a particular molecule binds relatively
specifically to a target molecule present in the tumor
following administration to a subject. In general,
selective homing is characterized, in part, by detecting
15   at least a two-fold (2x) greater specific binding of the
molecule to the tumor as compared to a control organ or
tissue.

It should be recognized that, in some cases, a
20   molecule can localize nonspecifically to an organ or
tissue containing a tumor. For example, in vivo panning
of a phage display library can result in high background
in organs such as liver and spleen, which contain a
marked component of the reticuloendothelial system (RES).
25   Thus, where a tumor is present, for example, in the
liver, nonspecific binding of molecules due to uptake by
the RES can make identifying a tumor homing molecule more
difficult.

30   Selective homing can be distinguished from
nonspecific binding, however, by detecting differences in
the abilities of different individual phage to home to a
tumor. For example, selective homing can be identified
by combining a putative tumor homing molecule such as a
35   peptide expressed on a phage with a large excess of
non-infective phage or with about a five-fold excess of
phage expressing unselected peptides, injecting the

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mixture into a subject and collecting a sample of the
tumor. In the latter case, for example, provided the
number of injected phage expressing tumor homing peptide
is sufficiently low so as to be nonsaturating for the
5   target molecule, a determination that greater than about
20% of the phage in the tumor express the putative tumor
homing molecule is demonstrative evidence that the
peptide expressed by the phage is a specific tumor homing
molecule. In addition, nonspecific localization can be
10   distinguished from selective homing by performing
competition experiments using, for example, phage
expressing a putative tumor homing peptide in combination
with an excess amount of the "free" peptide (Example IV).

15   In addition, various methods can be used to
prevent nonspecific binding of a molecule to an organ
containing a component of the RES. For example, a
molecule that homes selectively to a tumor present in an
organ containing a component of the RES can be obtained
20   by first blocking the RES using, for example, polystyrene
latex particles or dextran sulfate (see Kalin et al.,
Nucl. Med. Biol. 20:171-174 (1993); Illum et al.,
J. Pharm. Sci. 75:16-22 (1986); Takeya et al., J. Gen.
Microbiol. 100:373-379 (1977) , each of which is
25   incorporated herein by reference), then administering the
library to the subject. For example, pre-administration
of dextran sulfate 500 or polystyrene microspheres prior
to administration of a test substance has been used to
block nonspecific uptake of the test substance by Kupffer
30   cells, which are the RES component of the liver (Illum et
al., supra, 1986). Similarly, nonspecific uptake of
agents by the RES has been blocked using carbon particles
or silica (Takeya et al., supra, 1977) or a gelatine
colloid (Kalin et al., supra, 1993). Thus, various
35   agents useful for blocking nonspecific uptake by the RES
are known and routinely used.

25

Nonspecific binding of phage to RES or to other sites also can be prevented by coinjecting, for example, mice with a specific phage display library together with the same phage made noninfective (Smith et al., *supra*,

5 1990, 1993). In addition, a peptide that homes to tumor in an organ containing an RES component can be identified by preparing a phage display library using phage that exhibit low background binding to the particular organ. For example, Merrill et al. (*Proc. Natl. Acad. Sci., USA*

10 93:3188-3192 (1996), which is incorporated herein by reference) selected lambda-type phage that are not taken up by the RES and, as a result, remain in the circulation for a prolonged period of time. A filamentous phage variant, for example, can be selected using similar

15 methods.

Selective homing can be demonstrated by determining the specificity of a tumor homing molecule for the tumor as compared to a control organ or tissue.

20 Selective homing also can be demonstrated by showing that molecules that home to a tumor, as identified by one round of *in vivo* panning, are enriched for tumor homing molecules in a subsequent round of *in vivo* panning. For example, phage expressing peptides that selectively home

25 to a melanoma tumor were isolated by *in vivo* panning, then were subjected to additional rounds of *in vivo* panning. Following a second round of screening, phage recovered from the tumor showed a 3-fold enrichment in homing to the tumor as compared to brain. Phage

30 recovered from the tumor after a third round of screening showed an average of 10-fold enrichment in homing to the tumor as compared to brain. Selective homing also can be demonstrated by showing that molecules that home to a selected tumor, as identified by one round of *in vivo*

35 panning, are enriched for tumor homing molecules in a subsequent round of *in vivo* panning.

Tumor homing molecules can be identified by *in vivo* panning using, for example, a mouse containing a transplanted tumor. Such a transplanted tumor can be, for example, a human tumor that is transplanted into
5   immunodeficient mice such as nude mice or a murine tumor that is maintained by passage in tissue culture or in mice. Due to the conserved nature of cellular receptors and of ligands that bind a particular receptor, it is expected that angiogenic vasculature and histologically
10 similar tumor cells in various species can share common cell surface markers useful as target molecules for a tumor homing molecule. Thus, the skilled artisan would recognize that a tumor homing molecule identified using, for example, *in vivo* panning in a mouse having a murine
15 tumor of a defined histological type such as a melanoma also would bind to the corresponding target molecule in a tumor in a human or other species. Similarly, tumors growing in experimental animals require associated neovascularization, just as that required for a tumor
20 growing in a human or other species. Thus, a tumor homing molecule that binds a target molecule present in the vasculature in a tumor grown in a mouse likely also can bind to the corresponding target molecule in the vasculature of a tumor in a human or other mammalian
25 subject. The general ability of a tumor homing molecule identified, for example, by homing to a human breast tumor, also to home to a human Kaposi's sarcoma or to a mouse melanoma indicates that the target molecules are shared by many tumors. Indeed, the results disclosed
30 herein demonstrate that the target molecules are expressed in the neovasculature, which is host tissue (see Examples IV and VII).

A tumor homing molecule identified using *in
35 vivo* panning in an experimental animal such as a mouse readily can be examined for the ability to bind to a corresponding tumor in a human patient by demonstrating,

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for example, that the molecule also can bind specifically to a sample of the tumor obtained from the patient. For example, the CDCRGDCFC (SEQ ID NO: 1) phage and NGR peptides have been shown to bind to blood vessels in
5   microscopic sections of human tumors, whereas little or no binding occurs in the blood vessels of nontumor tissues. Thus, routine methods can be used to confirm that a tumor homing molecule identified using *in vivo* panning in an experimental animal also can bind the
10 target molecule in a human tumor.

The steps of administering the library to the subject, collecting a selected tumor and identifying the molecules that home to the tumor, comprise a single round
15 of *in vivo* panning. Although not required, one or more additional rounds of *in vivo* panning generally are performed. Where an additional round of *in vivo* panning is performed, the molecules recovered from the tumor in the previous round are administered to a subject, which
20 can be the same subject used in the previous round, where only a part of the tumor was collected.

By performing a second round of *in vivo* panning, the relative binding selectivity of the
25 molecules recovered from the first round can be determined by administering the identified molecules to a subject, collecting the tumor, and determining whether more phage is recovered from the tumor following the second round of screening as compared to those recovered
30 following the first round. Although not required, a control organ or tissue also can be collected and the molecules recovered from the tumor can be compared with those recovered from the control organ. Ideally, no molecules are recovered from a control organ or tissue
35 following a second or subsequent round of *in vivo* panning. Generally, however, a proportion of the molecules also will be present in a control organ or

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tissue. In this case, the ratio of molecules in the
selected tumor as compared to the control organ
(selected:control) can be determined. For example, phage
that homed to melanoma following a first round of *in vivo*

5   panning demonstrated a 3x enrichment in homing to the
selected tumor as compared to the control organ, brain,
following two additional rounds of panning (Example V).

Additional rounds of *in vivo* panning can be
10   used to determine whether a particular molecule homes
only to the selected tumor or can recognize a target on
the tumor that also is expressed in one or more normal
organs or tissues in a subject or is sufficiently similar
to the target molecule on the tumor. It is unlikely that
15   a tumor homing molecule also will home to a corresponding
normal tissue because the method of *in vivo* panning
selects only those molecules that home to the tumor,
which is selected. Where a tumor homing molecule also
directs homing to one or more normal organs or tissues in
20   addition to the tumor, the organs or tissues are
considered to constitute a family of selected organs or
tissues. Using the method of *in vivo* panning, molecules
that home to only the selected tumor can be distinguished
from molecules that also home to one or more selected
25   organs or tissues. Such identification is expedited by
collecting various organs or tissues during subsequent
rounds of *in vivo* panning.

The term "control organ or tissue" is used to
30   mean an organ or tissue other than the tumor for which
the identification of a tumor homing molecule is desired.
A control organ or tissue is characterized in that a
tumor homing molecule does not selectively home to the
control organ. A control organ or tissue can be
35   collected, for example, to identify nonspecific binding
of the molecule or to determine the selectivity of homing
of the molecule. In addition, nonspecific binding can be

identified by administering, for example, a control molecule, which is known not to home to a tumor but is chemically similar to a potential tumor homing molecule. Alternatively, were the administered molecules are linked
5 to a support, administration of the supports, alone, also can be used to identify nonspecific binding. For example, a phage that expresses the gene III protein, alone, but that does not contain a peptide fusion protein, can be studied by *in vivo* panning to determine
10 the level of nonspecific binding of the phage support.

As disclosed herein, specific homing of a tumor homing molecule readily can be identified by examining the selected tumor tissue as compared to a corresponding
15 nontumor tissue, as well as to control organs or tissues. For example, immunohistological analysis can be performed on a tumor tissue and corresponding nontumor tissue using an antibody specific for a phage used to display tumor homing peptides (see Example IV). Alternatively, an
20 antibody can be used that is specific for a shared tag that expressed with the peptide, for example, a FLAG epitope or the like, such detection systems being commercially available.

25 In general, a library of molecules, which contains a diverse population of random or selectively randomized molecules of interest, is prepared, then administered to a subject. At a selected time after administration, the subject is sacrificed and the tumor
30 is collected such that the molecules present in the tumor can be identified (see Example I). If desired, one or more control organs or tissues or a part of a control organ or tissue can be sampled. For example, mice bearing a breast tumor or a melanoma tumor were injected
35 with a phage peptide display library, then, after about 1 to 5 minutes, the mice were anesthetized, either frozen in liquid nitrogen or, preferably, are perfused through

the heart to terminate circulation of the phage, the
tumor and one or more control organs were collected from
each, phage present in the tumor and the control organs
were recovered and peptides that selectively homed to the
5   respective tumors were identified (see Examples I, II
and V).

        In the examples provided, the animals were
sacrificed to collect the selected tumor and control
10   organ or tissue. It should be recognized, however, that
only a part of a tumor need be collected to recover a
support containing a molecule that homes to that tumor
and, similarly, only part of an organ or tissue need be
collected as a control. Thus, a part of a tumor, for
15   example, can be collected by biopsy, such that a molecule
such as a peptide expressed by a phage can be
administered to the same subject a second time or more,
as desired. Where the molecule that is to be
administered a second time to the same subject is tagged
20   or linked, for example, to a support, the tag or support
should be nontoxic and biodegradable, so as not to
interfere with subsequent rounds of screening.

        In vitro screening of phage libraries
25   previously has been used to identify peptides that bind
to antibodies or to cell surface receptors (Smith and
Scott, *supra*, 1993). For example, *in vitro* screening of
phage peptide display libraries has been used to identify
novel peptides that specifically bound to integrin
30   adhesion receptors (Koivunen et al., J. Cell Biol.
124:373-380 (1994a), which is incorporated herein by
reference) and to the human urokinase receptor (Goodson
et al., Proc. Natl. Acad. Sci., USA 91:7129-7133 (1994)).
However, such *in vitro* studies provide no insight as to
35   whether a peptide that can specifically bind to a
selected receptor *in vitro* also will bind the receptor *in
vivo* or whether the binding peptide or the receptor are

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unique to a specific organ in the body. Furthermore, the
in vitro methods are performed using defined,
well-characterized target molecules in an artificial
system. For example, Goodson et al. (*supra*, 1994)

5   utilized cells expressing a recombinant urokinase
receptor. However, such *in vitro* methods are limited in
that they require prior knowledge of the target molecule
and yield little if any information regarding *in vivo*
utility.

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In vitro panning against cells in culture also
has been used to identify molecules that can specifically
bind to a receptor expressed by the cells (Barry et al.,
Nature Med. 2:299-305 (1996), which is incorporated

15   herein by reference). However, the cell surface
molecules that are expressed by a cell *in vivo* often
change when the cell is grown in culture. Thus, *in vitro*
panning methods using cells in culture also are limited
in that there is no guarantee a molecule that is

20   identified due to its binding to a cell in culture will
have the same binding ability *in vivo*. Furthermore, it
is not possible using *in vitro* panning to distinguish
molecules that home only to the tumor cells used in the
screening, but not to other cell types.

25

In contrast, *in vivo* panning requires no prior
knowledge or availability of a target molecule and
identifies molecules that bind to cell surface target
molecules that are expressed *in vivo*. Also, since the

30   "nontargeted" tissues are present during the screening,
the probability of isolating tumor homing molecules that
lack specificity of homing is greatly reduced.
Furthermore, in obtaining tumor homing molecules by *in
vivo* panning, any molecules that may be particularly

35   susceptible to degradation in the circulation *in vivo*
due, for example, to a metabolic activity, are not
recovered. Thus, *in vivo* panning provides significant

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advantages over previous methods by identifying molecules
that selectively home *in vivo* and the target molecule
present in a tumor.

5   Although mechanisms by which the disclosed
method of *in vivo* panning works have not been fully
defined, one possibility is that a molecule such as a
peptide expressed on a phage recognizes and binds to a
target molecule present on endothelial cells lining the
10   blood vessels in a tumor. Evidence indicates, for
example, that the vascular tissues in various organs
differ from one another and that such differences can be
involved in regulating cellular trafficking in the body.
For example, lymphocytes home to lymph nodes or other
15   lymphoid tissues due, in part, to the expression of
specific "address" molecules by the endothelial cells in
those tissues (Salmi et al., Proc. Natl. Acad. Sci., USA
89:11436-11440 (1992); Springer, Cell 76:301-314 (1994)).
Similarly, various leukocytes can recognize sites of
20   inflammation due, in part, to the expression of
endothelial cell markers induced by inflammatory signals
(see Butcher and Picker, Science 272:60-66 (1996);
Springer, *supra*, 1994). Thus, endothelial cell markers
provide a potential target for directing, for example, a
25   drug, which can be linked to a tumor homing molecule, to
a tumor in a subject.

           In some cases, the metastasis of cancer cells
to specific organs also can be due to recognition by the
30   tumor cell of an organ specific marker, including organ
specific endothelial cell markers (Fidler and Hart,
Science 217:998-1003 (1982)). The pattern of metastasis
of many cancers can be explained by assuming that
circulating tumor cells are preferentially trapped in the
35   first vascular bed encountered. Thus, the lungs and the
liver are the most frequent sites of cancer metastasis.
However, some cancers show patterns of metastasis that

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are not explained by circulatory routing. Metastasis of such cancers may be due to the presence of selectively expressed address molecules such as endothelial cell surface molecules expressed in the organ to which the

5 cancer metastasizes (see Goetz et al., Int. J. Cancer 65:192-199 (1996); Zhu et al., Proc. Natl. Acad. Sci., USA 88:9568-9572 (1991); Pauli et al., Cancer Metast. Rev. 9:175-189 (1990); Nicolson, Biochim. Biophys. Acta 948:175-224 (1988)). The identification of molecules

10 that bind to such organ-specific endothelial cell markers can provide a means to prevent tumor cell metastasis to the particular organ.

    The vasculature within a tumor generally

15 undergoes active angiogenesis, resulting in the continual formation of new blood vessels to support the growing tumor. Such angiogenic blood vessels are distinguishable from mature vasculature in that angiogenic vasculature expresses unique endothelial cell surface markers,

20 including the $\alpha_v\beta_3$ integrin (Brooks, Cell 79:1157-1164 (1994); WO 95/14714, Int. Filing Date November 22, 1994) and receptors for angiogenic growth factors (Mustonen and Alitalo, J. Cell Biol. 129:895-898 (1995); Lappi, Semin. Cancer Biol. 6:279-288 (1995)). Moreover, tumor

25 vasculature is histologically distinguishable from blood vessel in general in that tumor vasculature is fenestrated (Folkman, Nature Med. 1:27-31 (1995); Rak et al., Anticancer Drugs 6:3-18 (1995)). Thus, the unique characteristics of tumor vasculature make it a

30 particularly attractive target for determining whether a molecule that homes specifically to a tumor can be identified by in vivo panning. Such a tumor homing molecule can be useful for directing an agent such as a chemotherapeutic drug to a tumor, while reducing the

35 likelihood the agent will have a toxic effect on normal, healthy organs or tissues (Example VII). Moreover, a molecule that homes selectively to tumor vasculature also

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may have use in targeting other types of neovasculature
such as that present in inflammatory, regenerating or
wounded tissues.

5   Using *in vivo* panning to a breast carcinoma, a
melanoma and a Kaposi's sarcoma, phage expressing various
peptides that selectively homed to tumors were identified
(see Tables 1, 2 and 3, respectively). Due to the large
size of the phage (300 nm or 900-1000 nm ? PLEASE CONFIRM
10 SIZE OF PHAGE) and the short time the phage were allowed
to circulate (3 to 5 min), it is unlikely that a
substantial number of phage would have exited the
circulatory system, particularly in the brain and kidney.
Tissue staining studies indicated that the tumor homing
15 molecules that were identified primarily homed to and
bound endothelial cell surface markers, which likely are
expressed in an organ-specific manner. These results
indicate that *in vivo* panning can be used to identify and
analyze endothelial cell specificities. Such an analysis
20 is not possible using endothelial cells in culture
because the cultured cells tend to lose their tissue-
specific differences (Pauli and Lee, Lab. Invest.
58:379-387 (1988)).

25   Although the conditions under which the *in vivo*
pannings were performed identified tumor homing peptides
that generally bind to endothelial cell markers, the
specific presence of phage expressing tumor homing
peptides also was observed in tumor parenchyma,
30 particularly at later times after administration of the
peptides (Example IV). These results demonstrate that
phage expressing peptides can pass through the blood
vessels in the tumor, possibly due to the fenestrated
nature of the blood vessels, and indicate that the *in
35 vivo* panning method can be useful for identifying target
molecules expressed by tumor cells, as well as target
molecules expressed by endothelial cells.

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Phage peptide display libraries were
constructed essentially as described Smith and Scott
(*supra*, 1993; see, also, Koivunen et al., Biotechnology
13:265-270 (1995); Koivunen et al., Meth. Enzymol.
5   245:346-369 (1994b), each of which is incorporated herein
by reference). Oligonucleotides encoding peptides having
substantially random amino acid sequences were
synthesized based on an "NNK" codon, wherein "N" is A, T,
C or G and "K" is G or T. "NNK" encodes 32 triplets,
10 which encode the twenty amino acids and an amber STOP
codon (Scott and Smith, *supra*, 1990). In some libraries,
at least one codon encoding cysteine also was included in
each oligonucleotide so that cyclic peptides could be
formed through disulfide linkages (Example I). The
15 oligonucleotides were inserted in frame with the sequence
encoding the gene III protein (gIII) in the vector fuse 5
such that a peptide-gIII fusion protein can be expressed.
Following expression, the fusion protein is expressed on
the surface of the phage containing the vector (Koivunen
20 et al., *supra*, 1994b; Smith and Scott, *supra*, 1993).

Following *in vivo* panning, the phage isolated
based on their ability to selectively home to human
breast carcinoma, mouse melanoma or human Kaposi's
25 sarcoma tumors displayed only a few different peptide
sequences (see Tables 1, 2 and 3, respectively). One of
the screenings revealed peptide sequences that contained
the arginine-glycine-aspartic acid (RGD) integrin
recognition sequence (Ruoslahti, Ann. Rev. Cell Devel.
30 Biol. 12:697 (1996)) in the context of a peptide
previously demonstrated to bind selectively to
α_v-containing integrins (Koivunen et al., *supra*, 1995;
WO 95/14714). The sequences of most of the remaining
tumor homing peptides did not reveal any significant
35 similarities with known ligands for endothelial cell
receptors. However, one of the tumor homing peptides
contained the asparagine-glycine-arginine (NGR) motif,

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which is a weak integrin binding motif similar to the
motifs present in integrin-binding peptides (Ruoslahti et
al., U.S. Patent No. 5,536,814, issued July 16, 1996,
which is incorporated herein by reference; see, also,
5   Koivunen et al., supra, 1994a). Other screenings have
revealed numerous NGR-containing peptides (see Table 1).
Despite the weak integrin binding ability of NGR
peptides, an integrin receptor may not be the target
molecule recognized by the NGR tumor homing peptides
10   exemplified herein (Example VII). As used herein, the
term "integrin" means a heterodimeric cell surface
adhesion receptor.

    The peptides expressed by the phage that homed
15   to the breast tumor included the peptides CGRECPRLCQSSC
(SEQ ID NO: 2) and CNGRCVSGCAGRC (SEQ ID NO: 3; see
Table 1; Example II). Similarly, tumor homing peptides,
including the peptides CDCRGDCFC (SEQ ID NO: 1) and
CGSLVRC (SEQ ID NO: 5), were identified from two other
20   phage libraries administered to breast tumor bearing mice
(Table 1). Some of these motifs, as well as novel one,
also were isolated in the screen with mouse melanoma and
human Kaposi's sarcoma (see Tables 2 and 3). These
results demonstrated that tumor homing molecules can be
25   identified using in vivo panning.

    Three main tumor homing motifs emerged. As
discussed above, one motif contained the sequence RGD
(Ruoslahti, supra, 1996) embedded in the peptide
30   structure, CDCRGDCFC (SEQ ID NO: 1), which is known to
bind selectively to α_v integrins (Koivunen et al., supra,
1995; WO 95/14714). Since the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are
markers of angiogenic vessels (Brooks et al., supra,
1994; Friedlander et al., Science 270:1500 (1995)), a
35   phage expressing the peptide CDCRGDCFC (SEQ ID NO: 1) was
examined for tumor targeting and, as disclosed herein,
homed to tumors in a highly selective manner (see Example

III). Furthermore, homing by the CDCRGDCFC (SEQ ID
NO: 1) phage was inhibited by coadministration of the
free CDCRGDCFC (SEQ ID NO: 1) peptide.

5   Another breast tumor homing peptide had the
sequence CNGRCVSGCAGRC (SEQ ID NO: 3), which contains the
NGR motif previously shown to have weak integrin binding
activity (Koivunen et al., J. Biol. Chem. 268:20205-20210
(1993); Koivunen et al., supra, 1994a; WO 95/14714).
10   Since an NGR containing peptide was identified, two
additional peptides, the linear peptide, NGRAHA (SEQ ID
NO: 6), and the cyclic peptide, CVLNGRMEC (SEQ ID NO: 7),
each of which contains the NGR motif, were examined for
tumor homing. Like the phage expressing CNGRCVSGCAGRC
15   (SEQ ID NO: 3), phage expressing NGRAHA (SEQ ID NO: 6) or
CVLNGRMEC (SEQ ID NO: 7) homed to the tumors.
Furthermore, tumor homing was not dependent on the tumor
type or on species, as the phage accumulated selectively
in human breast carcinoma, as well as in the tumors of
20   mice bearing a mouse melanoma and mice bearing a human
Kaposi's sarcoma xenograft.

          The various peptides, including RGD- and
NGR-containing peptides, generally were bound to the
25   tumor blood vessels. The minimal cyclic NGR peptide,
CNGRC (SEQ ID NO: 8), was synthesized based on the
CNGRCVSGCAGRC (SEQ ID NO: 3) sequence. When the CNGRC
(SEQ ID NO: 8) peptide was co-injected with phage
expressing either CNGRCVSGCAGRC (SEQ ID NO: 3), NGRAHA
30   (SEQ ID NO: 6) or CVLNGRMEC (SEQ ID NO: 7), accumulation
of the phage in the breast carcinoma xenografts was
inhibited. However, the CNGRC (SEQ ID NO: 8) peptide did
not inhibit the homing of phage expressing the CDCRGDCFC
(SEQ ID NO: 1) peptide, even when administered in amounts
35   up to ten times higher than those that inhibited the
homing of the NGR phage. In comparison, the CDCRGDCFC
(SEQ ID NO: 1) peptide partially inhibited the homing of

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the NGR phage, although the amount needed was 5 to
10 fold higher than that of the CNGRC peptide (SEQ ID
NO: 8). These results indicate that NGR peptides and RGD
peptides bind to different receptor sites in tumor
5   vasculature.

        A third motif, GSL (glycine-serine-leucine),
also was identified following *in vivo* panning in mice
bearing breast carcinoma, malignant melanoma or Kaposi's
10 sarcoma. Homing of phage expressing the GSL peptide,
CGSLVRC (SEQ ID NO: 5), was inhibited by coadministration
of the free CGSLVRC (SEQ ID NO: 5) peptide. Like the RGD
and NGR peptides, phage expressing GSL peptides also
bound to blood vessels of tumors. In view of the
15 identification of the conserved RGD, NGR and GSL motifs
present in tumor homing peptides, as disclosed herein, it
will be recognized that peptides containing such motifs
can be useful as tumor homing peptides and, in
particular, for forming conjugates that can target a
20 moiety such as a cancer chemotherapeutic agent or a
diagnostic agent to a tumor.

        Various peptide libraries containing up to
13 amino acids were constructed and the NGR peptide,
25 CNGRCVSGCAGRC (SEQ ID NO: 3), was obtained as a result of
in vivo panning against a breast tumor. This NGR
peptide, which was obtained by screening a random peptide
library, was a tumor homing peptide (see Example VII).
In addition, when a peptide library was constructed based
30 on the formula CXXXNGRXX (SEQ ID NO: 13) or CXXCNGRCX
(SEQ ID NO: 14), each of which is biased toward NGR
sequences, and used for *in vivo* panning against a breast
tumor, numerous NGR peptides were obtained (see Table 1).

35   These results indicate that a tumor homing
peptide of the invention can comprise the amino acid
sequence RGD or NGR or GSL. Such tumor peptides can be

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as small as five amino acids, such as CNGRC (SEQ ID NO:
8). Such tumor homing peptides also can be not only at
least 13 amino acids in length, which is the largest
peptide exemplified herein, but can be up to 20 amino

5   acids, or 30 amino acids, or 50 to 100 amino acids in
length, as desired. A tumor homing peptide of the
invention conveniently is produced by chemical synthesis.

Immunohistochemical analysis was performed by

10   comparing tissue staining for phage allowed to circulate
for about four minutes, followed by perfusion through the
heart of the mice, or with tissues analyzed 24 hours
after phage injection (see Figure 1). At 24 hours
following administration, essentially no phage remain in

15   the circulation and, therefore, perfusion is not required
(Pasqualini et al., *supra*, 1997). Strong phage staining
was observed in tumor vasculature, but not in normal
endothelium, in samples examined four minutes after
administration of the CNGRCVSGCAGRC (SEQ ID NO: 3) phage

20   (Example IV; compare Figures 1E, 1G, 1H and 1J). In
comparison, staining of the tumor was strong at 24 hours
and appeared to have spread outside the blood vessels
into the tumor parenchyma (compare Figures 1A to 1D
and 1F (tumor) with Figures 1I and 1K to 1V (nontumor)).

25   The NGRAHA (SEQ ID NO: 6) and CVLNGRMEC (SEQ ID NO: 7)
phage showed similar staining patterns (Example IV). In
contrast, the control organs and tissues showed little or
no immunostaining, confirming the specificity of the NGR
motifs for tumor vessels. Spleen and liver, however,

30   captured phage, as expected, since uptake by the
reticuloendothelial system is a general property of phage
particles, independent of the presence of peptide
expression by the phage (Pasqualini et al., *supra*, 1997).

35   Immunostaining also was observed following
administration of phage expressing the GSL motif
containing peptide, CLSGSLSC (SEQ ID NO: 4), and, like

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that of the NGR peptides, was localized to the blood
vessels, in this case, within a melanoma tumor (see
below; see, also, Examples IV and V). Similarly,
immunostaining following administration of phage

5   expressing the RGD motif containing peptide, CDCRGDCFC
(SEQ ID NO: 1), to breast tumor bearing mice was
localized to the blood vessels in the tumor, but was not
observed in brain, kidney or various other nontumor
tissues (see Examples III and IV; see, also, Pasqualini

10 et al., *supra*, 1997). These results demonstrate that the
various tumor homing peptides generally home to tumor
vasculature.

The general applicability of the *in vivo*
15 panning method for identifying molecules that home to a
tumor was examined by injecting mice bearing a syngeneic
melanoma with phage expressing a diverse population of
peptides (Example V). The B16 mouse melanoma model was
selected for these studies because the tumors that form

20 are highly vascularized and because the biology of this
tumor line has been thoroughly characterized (see Miner
et al., Cancer Res. 42:4631-4638 (1982)). Furthermore,
because the B16 melanoma cells are of mouse origin,
species differences between the host and the tumor cell

25 donor will not affect, for example, the distribution of
phage into the tumor as compared to into normal organs.
As disclosed herein, *in vivo* panning against B16 melanoma
cells revealed tumor homing peptides, including, for
example, the GSL moiety containing peptide CLSGSLSC (SEQ

30 ID NO: 4; see, also, Table 2) and immunohistochemical
staining of the tumor and other organs using an
anti-phage antibody demonstrated that the CLSGSLSC (SEQ
ID NO: 4) expressing phage resulted in immunostaining in
the melanoma, but essentially no staining in skin, kidney

35 or other control organs (Example V). The staining
pattern generally followed the blood vessels within the

melanoma, but was not strictly confined to the blood vessels.

Although *in vivo* panning was performed in mice, at least the peptides comprising an NGR, RGD or GSL motif also likely can target human vasculature. The NGR phage binds to blood vessels in the transplanted human breast tumor, but not to blood vessels in normal tissues, indicating that this motif can be particularly useful for tumor targeting in patients. The CDCRGDCFC (SEQ ID NO: 1) peptide binds to human α_v-integrins (Koivunen et al., *supra*, 1995), which are selectively expressed in tumor blood vessels of human patients (Max et al., Int. J. Cancer 71:320 (1997); Max et al., Int. J. Cancer 72:706 (1997)). Use of a moiety/CDCRGDCFC (SEQ ID NO: 1) conjugate to target the moiety to a tumor also provides the additional advantage that the moiety will be targeted to tumor cells, themselves, because breast carcinoma cells, for example, can express the $\alpha_v\beta_3$ integrin (Pasqualini et al., *supra*, 1997). In fact, many human tumors express this integrin, which may be involved in the progression of certain tumors such as malignant melanomas (Albelda et al., Cancer Res. 50:6757-6764 (1990); Danen et al., Int. J. Cancer 61:491-496 (1995); Felding-Habermann et al., J. Clin. Invest. 89:2018-2022 (1992); Sanders et al., Cold Spring Harb. Symp. Quant. Biol. 58:233-240 (1992); Mitjans et al., J. Cell. Sci. 108:3067-3078 (1995)). Unlike the CDCRGDCFC (SEQ ID NO: 1) peptide, the NGR peptides do not appear to bind to MDA-MD-435 breast carcinoma cells. However, NGR peptides were able to deliver a therapeutically effective amount of doxorubicin to breast tumors (Example VII), indicating that, even where a tumor homing molecule homes only to tumor vasculature, i.e., not directly to the tumor cells, such vasculature targeting in sufficient to confer the effect of the moiety linked to the molecule.

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Since the $\alpha_v\beta_3$ integrin is expressed by endothelial cells in angiogenic vasculature, experiments were performed to determine whether tumor vasculature that is undergoing angiogenesis can be targeted *in vivo*

5   using methods as disclosed herein. Phage expressing the peptide, CDCRGDCFC (SEQ ID NO: 1; see, Koivunen et al., *supra*, 1995), which is known to bind to the $\alpha_v\beta_3$ integrin, were injected into mice bearing tumors formed from human breast carcinoma cells, mouse melanoma cells or human

10 Kaposi's sarcoma cells (see Example IV). The CDCRGDCFC (SEQ ID NO: 1) phage selectively homed to each of the tumors, whereas such homing did not occur with control phage. For example, in mice bearing tumors formed by implantation of human breast carcinoma cells, a twenty-

15 to eighty-fold greater number of the CDCRGDCFC (SEQ ID NO: 1) phage, as compared to unselected control phage, accumulated in the tumor.

Tissue staining for the phage showed

20 accumulation of the CDCRGDCFC (SEQ ID NO: 1) phage in the blood vessels within the tumor, whereas no staining was observed in brain, kidney or other control organs. Specificity of tumor homing by the CDCRGDCFC (SEQ ID NO: 1) phage was demonstrated by competition experiments,

25 in which coinjection of the free CDCRGDCFC (SEQ ID NO: 1) peptide greatly reduced tumor homing of the RGD phage, whereas coinjection of a non-RGD-containing control peptide had no effect on homing of the RGD phage (see Example III). These results demonstrate that the $\alpha_v\beta_3$

30 target molecule is expressed on the luminal surface of endothelial cells in a tumor and that a peptide that binds to an α_v-containing integrin can bind selectively to this integrin and, therefore, to vasculature undergoing angiogenesis.

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The results of these studies indicate that tumor homing molecules can be identified by *in vivo*

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panning and that, in some cases, a tumor homing molecule can home to vascular tissue in the tumor as well as to tumor parenchyma, probably due to the fenestrated nature of the blood vessels permitting ready exit of the phage
5   from the circulatory system. Due to the ability of such tumor homing molecules to home to tumors, the molecules are useful for targeting a linked moiety to tumors. Thus, the invention provides conjugates comprising a tumor homing molecule linked to a moiety, such conjugates
10   being useful for targeting the moiety to tumor cells.

The ability of a molecule that homes to a particular tumor to selectively home to another tumor of the same or a similar histologic type can be determined
15   using, for example, human tumors grown in nude mice or mouse tumors grown in syngeneic mice for these experiments. For example, various human breast cancer cell lines, including MDA-MB-435 breast carcinoma (Price et al., Cancer Res. 50:717-721 (1990)), SKBR-1-II and
20   SK-BR-3 (Fogh et al., J. Natl. Cancer Inst. 59:221-226 (1975)), and mouse mammary tumor lines, including EMT6 (Rosen et al., Int. J. Cancer 57:706-714 (1994)) and C3-L5 (Lala and Parhar, Int. J. Cancer 54:677-684 (1993)), are readily available and commonly used as
25   models for human breast cancer. Using such breast tumor models, for example, information relating to the specificity of an identified breast tumor homing molecule for diverse breast tumors can be obtained and molecules that home to a broad range of different breast tumors or
30   provide the most favorable specificity profiles can be identified. In addition, such analyses can yield new information, for example, about tumor stroma, since stromal cell gene expression, like that of endothelial cells, can be modified by the tumor in ways that cannot
35   be reproduced *in vitro*.

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Selective homing of a molecule such as a
peptide or protein to a tumor can be due to specific
recognition by the peptide of a particular cell target
molecule such as a cell surface receptor present on a
5   cell in the tumor. Selectivity of homing is dependent on
the particular target molecule being expressed on only
one or a few different cell types, such that the molecule
homes primarily to the tumor. As discussed above, the
identified tumor homing peptides, at least in part, can
10 recognize endothelial cell surface markers in the blood
vessels present in the tumors. However, most cell types,
particularly cell types that are unique to an organ or
tissue, can express unique target molecules. Thus, *in
vivo* panning can be used to identify molecules that
15 selectively home to a particular type of tumor cell such
as a breast cancer cell and specific homing can be
demonstrated by performing the appropriate competition
experiments.

20   A tumor homing molecule of the invention can be
used to target a moiety to a tumor by linking the moiety
to the molecule to produce a tumor homing molecule/moiety
conjugate and administering the conjugate to a subject
having a tumor. As used herein, the term "moiety" is
25 used broadly to mean a physical, chemical, or biological
material that is linked to a tumor homing molecule for
the purpose of being targeted *in vivo* to a tumor or to
angiogenic vasculature expressing a target molecule
recognized by the tumor homing molecule. In particular,
30 a moiety is a biologically useful moiety such as
therapeutic moiety, a diagnostic moiety or a drug
delivery vehicle. Thus, a moiety can be a therapeutic
agent, for example, a cancer chemotherapeutic agent such
as doxorubicin, which, when linked to a tumor homing
35 molecule, provides a conjugate useful for treating a
cancer in a subject. In addition, a moiety can be a drug
delivery vehicle such as a chambered microdevice, a cell,

a liposome or a virus, which can contain an agent such as a drug or a nucleic acid.

A moiety also can be a molecule such as a
5   polypeptide or nucleic acid, to which a tumor homing
molecule is grafted for the purpose of directing the
polypeptide or nucleic acid to a selected tumor (Smith et
al., *J. Biol. Chem.* 269:32788-32795 (1994); Goldman et
al., *Cancer Res.* 15:1447-1451 (1997), each of which is
10 incorporated herein by reference). For example, a
peptide tumor homing molecule can be expressed as a
fusion protein with a desired polypeptide such that the
peptide targets the grafted polypeptide to a selected
tumor. Such a desired polypeptide, which is grafted to
15 the tumor homing peptide, can be a polypeptide involved
in initiating a cell death pathway, for example,
caspase 8, thus providing a means to direct caspase 8 to
a tumor, where it can induce apoptosis of the tumor cells
or of the vasculature supplying the tumor. A tumor
20 homing peptide also can be grafted to a polypeptide
expressed by a virus, for example, the adenovirus penton
base coat protein, thus providing a means to target a
virus to a tumor (Wickham et al., *Gene Ther.* 2:750-756
(1995); Weitzman et al., In: "Gene Therapy and Vector
25 Systems" 2:17-25 (1997), each of which is incorporated
herein by reference; see, also, Example III). Such a
grafted virus can contain an exogenous gene useful in a
method of gene therapy. Accordingly, the invention
provides compositions of matter comprising a tumor homing
30 molecule/moiety conjugate.

A moiety can be a detectable label such a
radiolabel or can be a cytotoxic agent, including a toxin
such as ricin or a drug such as a chemotherapeutic agent
35 or can be a physical, chemical or biological material
such as a liposome, microcapsule, micropump or other
chambered microdevice, which can be used, for example, as

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a drug delivery system. Generally, such microdevices, should be nontoxic and, if desired, biodegradable. Various moieties, including microcapsules, which can contain an agent, and methods for linking a moiety,

5 including a chambered microdevice, to a molecule of the invention are well known in the art and commercially available (see, for example, "Remington's Pharmaceutical Sciences" 18th ed. (Mack Publishing Co. 1990), chapters 89-91; Harlow and Lane, Antibodies: A laboratory manual

10 (Cold Spring Harbor Laboratory Press 1988), each of which is incorporated herein by reference; see, also, Hermanson, supra, 1996).

As disclosed herein, a moiety can be, for

15 example, a cancer chemotherapeutic agent linked to a tumor homing molecule to produce a tumor homing molecule/moiety conjugate. Cytotoxic chemotherapy is the basis of the systemic treatment of disseminated malignant tumors. However, a major limitation of the currently

20 used chemotherapeutic agents is that these drugs have the narrowest therapeutic index in all of medicine. As such, the dose of cancer chemotherapeutic agents generally is limited by undesirable toxicity to the patient being treated. Thus, the ability of tumor homing peptides of

25 the invention to target drugs into tumors was examined. As disclosed herein, the linking of a cancer chemotherapeutic agent, doxorubicin, to a tumor homing molecule reduced the systemic toxicity of the doxorubicin and enhanced anti-tumor activity of the agent (see

30 Example VII).

A conjugate of the invention is exemplified herein by doxorubicin linked to various tumor homing peptides (see Examples VI and VII). In view of the

35 exemplified method of linking doxorubicin to various tumor homing peptides and the disclosed efficacy of such conjugates of the invention, the skilled artisan will

recognize that various other chemotherapeutic agents also can be linked to a tumor homing molecule to make a conjugate of the invention. Cancer chemotherapeutic agents have been linked to antibodies, for example, for

5 the purpose of targeting the agents to cells such as tumor cells that express the antigen recognized by the antibodies. In addition, in such antibody/drug conjugates, the agent can maintain its therapeutic function and the antibody can maintain its antigen

10 binding specificity. For example, the anthracyclin, doxorubicin, has been linked to antibodies and the antibody/doxorubicin conjugates have been therapeutically effective in treating tumors (Sivam et al., Cancer Res. 55:2352-2356 (1995); Lau et al., Bioorg. Med. Chem.

15 3:1299-1304 (1995); Shih et al., Cancer Immunol. Immunother. 38:92-98 (1994)). Similarly, other anthracyclins, including idarubicin and daunorubicin, have been chemically conjugated to antibodies, which have delivered effective doses of the agents to tumors

20 (Rowland et al., Cancer Immunol. Immunother. 37:195-202 (1993); Aboud-Pirak et al., Biochem. Pharmacol. 38:641-648 (1989)).

In addition to the anthracyclins, alkylating

25 agents such as melphalan and chlorambucil have been linked to antibodies to produce therapeutically effective conjugates (Rowland et al., supra, 1994; Smyth et al., Immunol. Cell Biol. 65:315-321 (1987)), as have vinca alkaloids such as vindesine and vinblastine (Aboud-Pirak

30 et al., supra, 1989; Starling et al., Bioconj. Chem. 3:315-322 (1992)). Similarly, conjugates of antibodies and antimetabolites such as 5-fluorouracil, 5-fluorouridine and derivatives thereof have been effective in treating tumors (Krauer et al., Cancer Res.

35 52:132-137 (1992); Henn et al., J. Med. Chem. 36:1570-1579 (1993)). Other chemotherapeutic agents, including cis-platinum (Schechter et al., Int. J. Cancer 48:167-172

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(1991)), methotrexate (Shawler et al., J. Biol. Resp. Mod. 7:608-618 (1988); Fitzpatrick and Garnett, Anticancer Drug Des. 10:11-24 (1995)) and mitomycin-C (Dillman et al., Mol. Biother. 1:250-255 (1989)) also are

5   therapeutically effective when administered as conjugates with various different antibodies.

The results obtained using antibody/drug conjugates demonstrate that a chemotherapeutic agent can

10   be linked to an antibody to produce a conjugate that maintains the antigen binding specificity of the antibody and the therapeutic function of the agent. As disclosed herein, a conjugate comprising doxorubicin and a tumor homing peptide maintains the tumor homing specificity of

15   the tumor homing peptide as well as the therapeutic efficacy of the chemotherapeutic agent (see Example VII). Such results are remarkable, since, in the doxorubicin/CNGRC (SEQ ID NO: 8) conjugate, for example, the doxorubicin component has only a slightly lower

20   molecular weight than the peptide and comprises about 46% of the molecular weight of the conjugate.

Since the moiety component of a tumor homing molecule/moiety conjugate can comprise a substantial

25   portion of the conjugate without adversely affecting the ability of the tumor homing molecule to home to a tumor, additional components can be included as part of the conjugate, if desired. For example, in some cases, it can be desirable to utilize an oligopeptide spacer

30   between a tumor homing peptide and the moiety (Fitzpatrick and Garnett, Anticancer Drug Des. 10:1-9 (1995)). In this way, panels of moiety/spacer complexes can be constructed, in which a common spacer is linked to various different moieties. Such panels of moiety/spacer

35   conjugates can facilitate linkage of the moiety to a tumor homing molecule such as a tumor homing peptide of choice.

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Doxorubicin is one of the most commonly used
cancer chemotherapeutic agents and, particularly, is used
for treating breast cancer (Stewart and Ratain, In:
"Cancer: Principles and practice of oncology" 5th ed.,
5 chap. 19 (eds. DeVita, Jr., et al.; J.P. Lippincott
1997); Harris et al., In "Cancer: Principles and practice
of oncology," supra, 1997). In addition, doxorubicin has
anti-angiogenic activity (Folkman, supra, 1997; Steiner,
In "Angiogenesis: Key principles-Science, technology and
10 medicine," pp. 449-454 (eds. Steiner et al.; Birkhauser
Verlag, 1992)), which can contribute to its effectiveness
in treating cancer. Thus, treatment of human breast
cancer xenografts in mice using doxorubicin was selected
as a model for exemplifying the present invention.
15

CDCRGDCFC (SEQ ID NO: 1) and CNGRC (SEQ ID
NO: 8) were coupled to doxorubicin (Example VI) and the
peptide/doxorubicin conjugates were used to treat mice
bearing tumors derived from human MDA-MB-435 breast
20 carcinoma cells (Example VII). Mice were treated with
5 μg/week of doxorubicin equivalent (i.e., either free
doxorubicin or the doxorubicin component of the
peptide/doxorubicin conjugate), as compared to the more
commonly used 50-200 μg/mouse used in tumor bearing mice
25 (Berger et al., In "The Nude Mouse in Oncology Research"
(CRC Press 1991)). The lower dose was selected because
it was expected that the conjugate would be more
effective than the free drug.

30   MDA-MB-435 tumor-bearing mice treated with the
doxorubicin/CDCRGDCFC (SEQ ID NO: 1) conjugate had
significantly smaller tumors, less spread to regional
lymph nodes, and fewer pulmonary metastasis than mice
treated with free doxorubicin (see Example VII). All of
35 the mice treated with the doxorubicin/CDCRGDCFC (SEQ ID
NO: 1) conjugate survived beyond the time when all of the
mice treated with free doxorubicin had died from

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widespread disease. In a dose-escalation experiment, the
tumor bearing mice were treated with the
doxorubicin/CDCRGDCFC (SEQ ID NO: 1) conjugate at
30 μg/mouse every three weeks for three cycles, then were
5   observed, without further treatment, for an extended
period of time. The conjugate treated mice all remained
alive more than 6 months after the control, doxorubicin
treated mice had died (Example VII). These results
indicate that primary tumor growth and metastasis
10   significantly were inhibited in mice treated with the
conjugate and that cures may have occurred.

Many of the mice that received
doxorubicin/CDCRGDCFC (SEQ ID NO: 1) conjugate presented
15   marked skin ulceration and tumor necrosis; no such signs
were observed in mice treated with free doxorubicin or
with doxorubicin conjugated to an unrelated peptide
(Example VII). Histopathological analysis disclosed a
pronounced destruction of the vasculature in the tumors
20   treated with conjugate as compared to mice treated with
free doxorubicin. Furthermore, when tumors were removed
from the mice and the tumor cells plated in culture,
viability of cells from the tumors of mice receiving the
doxorubicin/CDCRGDCFC (SEQ ID NO: 1) conjugate was about
25   3 fold less than cells from tumors of mice treated with
the free doxorubicin (see Example VII). These results
demonstrate that administration to a tumor bearing mouse
of a conjugate comprising a chemotherapeutic agent linked
to a tumor homing molecule is more efficacious than
30   administration of the agent, alone, in treating a tumor.

Toxicity was determined by administration of
200 μg/doxorubicin equivalent in mice with very large,
size matched breast tumors. All of the mice treated with
35   the doxorubicin/CDCRGDCFC (SEQ ID NO: 1) conjugate
survived more than a week, while all of the mice treated

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with free doxorubicin died within 48 hours of the
administration of the drug (Example VII). These results
indicate that accumulation of the tumor homing
peptide/doxorubicin conjugate in the large tumors can
5   reduce systemic toxicity of the agent.

     Similar toxicity and treatment efficacy results
were obtained when breast tumor bearing mice were treated
using a doxorubicin/CNGRC (SEQ ID NO: 8) conjugate.
10   Tumors in the mice treated with the CNGRC (SEQ ID NO: 8)
conjugate were significantly smaller than in the control
groups; the conjugate suppressed tumor growth almost
completely. A strong effect on survival also occurred.
Free doxorubicin or doxorubicin conjugated to an
15   unrelated peptide, at the dose used, had little if any
effect on tumor growth relative to vehicle alone.

     Cytotoxic activity of free doxorubicin and the
doxorubicin/peptide conjugates was compared *in vitro*
20   using MDA-MB-435 cells. When cells were exposed to free
doxorubicin, doxorubicin/CDCRGDCFC (SEQ ID NO: 1) or
doxorubicin conjugated to an unrelated peptide for
30 minutes, cell death occurred only in the cultures
treated with the doxorubicin/CDCRGDCFC (SEQ ID NO: 1)
25   conjugate. In comparison, cells were killed by all of
the treatments after 24 hours of exposure. These results
indicate that enhanced cellular uptake of the
doxorubicin/CDCRGDCFC (SEQ ID NO: 1) conjugate occurs.

30   As disclosed herein, tumor homing molecules of
the invention can bind to the endothelial lining of small
blood vessels of tumors. The vasculature within tumors
is distinct, presumably due to the continual
neovascularization, resulting in the formation of new
35   blood vessels required for tumor growth. The distinct
properties of the angiogenic neovasculature within tumors
are reflected in the presence of specific markers in

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endothelial cells and pericytes (Folkman, Nature
Biotechnol. 15:510 (1997); Risau, FASEB J. 9:926-933
(1995); Brooks et al., *supra*, 1994); these markers likely
are being targeted by the tumor homing molecules of the
5   invention.

The ability of a tumor homing molecule to
target the blood vessels in a tumor provides substantial
advantages over methods of systemic treatment or methods
10   that directly target the tumor cells. For example, tumor
cells depend on a vascular supply for survival and the
endothelial lining of blood vessels is readily accessible
to a circulating probe. Conversely, in order to reach
solid tumor cells, a chemotherapeutic agent must overcome
15   potentially long diffusion distances, closely packed
tumor cells, and a dense fibrous stroma with a high
interstitial pressure that impedes extravasation (Burrows
and Thorpe, Pharmacol. Ther. 64:155-174 (1994)).

20   In addition, where the tumor vasculature is
targeted, the killing of all target cells may not be
required, since partial denudation of the endothelium can
lead to the formation of an occlusive thrombus halting
the blood flow through the entirety of the affected tumor
25   vessel (Burrows and Thorpe, *supra*, 1994). Furthermore,
unlike direct tumor targeting, there is an intrinsic
amplification mechanism in tumor vasculature targeting.
A single capillary loop can supply nutrients to up to
100 tumor cells, each of which is critically dependent on
30   the blood supply (Denekamp, Cancer Metast. Rev. 9:267-282
(1990); Folkman, *supra*, 1997).

Endothelial cells in a tumor also are unlikely
to lose a cell surface target receptor or develop a drug
35   resistance phenotype, as can develop through mutation and
clonal evolution of tumor cells, because endothelial
cells are genetically stable despite their high

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proliferation rates (Burrows and Thorpe, *supra*, 1994; Folkman, *supra*, 1995; Folkman, *supra*, 1997). In this regard, it has been long recognized by medical oncologists that, while tumors treated with

5 chemotherapeutic agents commonly develop drug resistance, normal tissues such as bone marrow do not develop such resistance. Thus, toxicity to normal tissues such as chemotherapy induced myelosuppression continues to occur during a treatment, even after tumor cells have become

10 drug resistant. Since the endothelial cells in blood vessels supplying a tumor are nontumor cells, it is expected that they will not develop resistance to chemotherapeutic agents, in a manner analogous to bone marrow cells. In fact, drug resistance has not been

15 observed during long term anti-angiogenic therapy in either experimental animals or in clinical trials (Folkman, *supra*, 1997).

Linking of a moiety larger than an agent such

20 as a drug or other organic or biologic molecule to a tumor homing molecule for the purpose of directing homing of the moiety to the selected tumor is exemplified by expressing an RGD-containing peptide on a phage, wherein the peptide directed homing of the phage to breast tumor

25 vasculature (Example IV). These results indicate that a tumor homing molecule of the invention can be linked to other moieties including, for example, a chambered microdevice or a liposome or a cell such as a white blood cell (WBC), which can be a cytotoxic T cell or a killer

30 cell, wherein upon administration of the tumor homing molecule/WBC conjugate, the molecule directs homing of the WBC to the tumor, where the WBC can exert its effector function.

35 The linking of a moiety to a tumor homing molecule can result in the molecule directing homing of the linked moiety to a tumor. For example, the linking

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of a brain homing peptide to a RBC directed homing of the
RBC to brain (see U.S. Patent No. 5,622,699; Pasqualini
and Ruoslahti, *supra*, 1996). This result indicates that
a tumor homing molecule of the invention also can be
5   linked to cell type or to a physical, chemical or
biological delivery system such as a liposome or other
encapsulating device, which can contain an agent such as
drug, in order to direct the cell type or the delivery
system to a selected tumor. For example, a tumor homing
10   molecule identified by *in vivo* panning can be linked to a
white blood cell (WBC) such as a cytotoxic T cell or a
killer cell, wherein upon administration of the tumor
homing molecule/WBC conjugate, the molecule directs
homing of the WBC to the tumor, where the WBC can exert
15   its effector function. Similarly, a tumor homing
molecule can be linked to a liposome or to a chambered
microdevice comprising, for example, a permeable or
semipermeable membrane, wherein an agent such as a drug
to be delivered to a selected tumor is contained within
20   the liposome or microdevice. Such compositions also can
be useful, for example, for delivering a nucleic acid
molecule to a tumor cells, thereby providing a means for
performing *in vivo* targeted gene therapy.

25   In one embodiment, a tumor homing molecule is
linked to a moiety that is detectable external to the
subject, thereby providing a composition useful to
perform an *in vivo* diagnostic imaging study. For
example, *in vivo* imaging using a detectably labeled tumor
30   homing peptide can identify the presence of a tumor in a
subject. For such studies, a moiety such as a gamma ray
emitting radionuclide, for example, indium-111 or
technitium-99, can be linked to the tumor homing molecule
and, following administration to a subject, can be
35   detected using a solid scintillation detector.
Similarly, a positron emitting radionuclide such as
carbon-11 or a paramagnetic spin label such as carbon-13

can be linked to the molecule and, following administration to a subject, the localization of the moiety/molecule can be detected using positron emission transaxial tomography or magnetic resonance imaging,

5   respectively. Such methods can identify a primary tumor as well as a metastatic lesion, which may not be detectable using other methods. Having identified the presence of a cancer in a subject, in another embodiment of the invention, the tumor homing molecule is linked to

10   a cytotoxic agent such as ricin or a cancer chemotherapeutic agent such as doxorubicin in order to direct the moiety to the tumor or can be linked to a chambered microdevice, which can contain a chemotherapeutic drug or other cytotoxic agent.● Use of

15   such a composition provides a means to selectively killing of the tumor, while substantially sparing normal tissues in a cancer patient and, therefore, the conjugates of the invention provide useful medicaments for diagnosing or treating a cancer patient.

20

        The skilled artisan would recognize that various tumor homing molecules can selectively home only to a tumor or can selectively home to a tumor and to a family of selected organs, including, in some cases, the

25   normal tissue counterpart to the tumor. Thus, the artisan would select a tumor homing peptide for administration to a subject based on the procedure being performed. For example, a tumor homing molecule that homes only to a tumor can be useful for directing a

30   therapy to the tumor. In comparison, a tumor homing molecule that selectively homes not only to the tumor, but also to one or more normal organs or tissues, can be used in an imaging method, whereby homing to an organ or tissue other than the tumor provides an internal imaging

35   control. Such an internal control can be useful, for example, for detecting a change in the size of a tumor in response to a treatment, since the normal organ is not

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expected to change in size and, therefore, can be compared with the tumor size.

Tumor homing peptides, which are identified by
5   *in vivo* panning, can be synthesized in required
quantities using routine methods of solid state peptide
synthesis or can be purchased from commercial sources
(for example, Anaspec; San Jose CA) and a desired moiety
can be linked to the molecule. Several methods useful
10   for linking a moiety to a molecule are known in the art,
depending on the particular chemical characteristics of
the molecule. For example, methods of linking haptens to
carrier proteins as used routinely in the field of
applied immunology (see, for example, Harlow and Lane,
15   *supra*, 1988; Hermanson, *supra*, 1996).

It is recognized that, in some cases, a drug
can lose cytotoxic efficacy upon conjugation or
derivatization depending, for example, on the conjugation
20   procedure or the chemical group utilized (Hurwitz et al.,
Cancer Res. 35:1175-1181 (1975); Trail et al., Science
261;212-215 (1993); Nagy et al., Proc. Natl. Acad. Sci.,
USA 93:7269-7273 (1996)). Moreover, it is recognized
that a phage that yields a tumor homing peptide of the
25   invention displays as many as five of the peptides.
Thus, there is a possibility that the affinity of an
individual peptide is too low for effective tumor homing
and that multivalent, rather than univalent, peptide
conjugates must be used. However, as disclosed herein,
30   doxorubicin maintained cytotoxic activity when used as a
conjugate with tumor homing peptides (see Example VII),
thus allaying the potential concerns discussed above.

A moiety such as a therapeutic or diagnostic
35   agent can be conjugated to a tumor homing peptide using,
for example, carbodiimide conjugation (Bauminger and
Wilchek, Meth. Enzymol. 70:151-159 (1980), which is

incorporated herein by reference). Carbodiimides
comprise a group of compounds that have the general
formula R-N=C=N-R', where R and R' can be aliphatic or
aromatic, and are used for synthesis of peptide bonds.
5   The preparative procedure is simple, relatively fast, and
is carried out under mild conditions. Carbodiimide
compounds attack carboxylic groups to change them into
reactive sites for free amino groups. Carbodiimide
conjugation has been used to conjugate a variety of
10   compounds to carriers for the production of antibodies.

     The water soluble carbodiimide, 1-ethyl-3-
(3-dimethylaminopropyl) carbodiimide (EDC) is
particularly useful for conjugating a moiety to a tumor
15   homing peptide and was used to conjugate doxorubicin to
tumor homing peptides (Example VI). The conjugation of
doxorubicin and a tumor homing peptide requires the
presence of an amino group, which is provided by
doxorubicin, and a carboxyl group, which is provided by
20   the peptide. EDC coupling of doxorubicin to the CNGRC
(SEQ ID NO: 8) peptide was performed using a 1:1 molar
ratio of the peptide (carboxyl groups) to obtain a
doxorubicin/CNGRC (SEQ ID NO: 8; see Example VI).

25   In addition to using carbodiimides for the
direct formation of peptide bonds, EDC also can be used
to prepare active esters such as N-hydroxysuccinimide
(NHS) ester. The NHS ester, which binds only to amino
groups, then can be used to induce the formation of an
30   amide bond with the single amino group of the
doxorubicin. The use of EDC and NHS in combination is
commonly used for conjugation in order to increase yield
of conjugate formation (Bauminger and Wilchek, *supra*,
1980).
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          Other methods for conjugating a moiety to a
tumor homing molecule also can be used. For example,

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sodium periodate oxidation followed by reductive
alkylation of appropriate reactants can be used, as can
glutaraldehyde crosslinking. However, it is recognized
that, regardless of which method of producing a conjugate
5   of the invention is selected, a determination must be
made that the tumor homing molecule maintains its
targeting ability and that the moiety maintains its
relevant function. Methods as disclosed in Example VII
or otherwise known in the art can confirm the activity of
10   the moiety/tumor homing molecule conjugate.

The yield of moiety/tumor homing molecule
conjugate formed is determined using routine methods.
For example, HPLC or capillary electrophoresis or other
15   qualitative or quantitative method can be used (see, for
example, Liu et al., J. Chromatogr. 735:357-366 (1996);
Rose et al., J. Chromatogr. 425:419-412 (1988), each of
which is incorporated herein by reference; see, also,
Example V). In particular, the skilled artisan will
20   recognize that the choice of a method for determining
yield of a conjugation reaction depends, in part, on the
physical and chemical characteristics of the specific
moiety and tumor homing molecule. Following conjugation,
the reaction products are desalted to remove any free
25   peptide and free drug.

When administered to a subject, the tumor
homing molecule/moiety conjugate is administered as a
pharmaceutical composition containing, for example, the
30   conjugate and a pharmaceutically acceptable carrier.
Pharmaceutically acceptable carriers are well known in
the art and include, for example, aqueous solutions such
as water or physiologically buffered saline or other
solvents or vehicles such as glycols, glycerol, oils such
35   as olive oil or injectable organic esters.

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A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize or to increase the absorption of the conjugate. Such physiologically acceptable
5   compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art would know that the
10 choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the composition. The pharmaceutical composition also can contain an agent such as a cancer therapeutic agent.
15

One skilled in the art would know that a pharmaceutical composition containing a tumor homing molecule can be administered to a subject by various routes including, for example, orally or parenterally,
20 such as intravenously. The composition can be administered by injection or by intubation. The pharmaceutical composition also can be a tumor homing molecule linked to liposomes or other polymer matrices, which can have incorporated therein, for example, a drug
25 such as a chemotherapeutic agent (Gregoriadis, Liposome Technology, Vol. 1 (CRC Press, Boca Raton, FL 1984), which is incorporated herein by reference). Liposomes, for example, which consist of phospholipids or other lipids, are nontoxic, physiologically acceptable and
30 metabolizable carriers that are relatively simple to make and administer.

For the diagnostic or therapeutic methods disclosed herein, an effective amount of the tumor homing
35 molecule/moiety conjugate must be administered to the subject. As used herein, the term "effective amount" means the amount of the conjugate that produces the

desired effect. An effective amount often will depend on
the moiety linked to the tumor homing molecule. Thus, a
lesser amount of a radiolabeled molecule can be required
for imaging as compared to the amount of a drug/molecule

5   conjugate administered for therapeutic purposes. An
effective amount of a particular molecule/moiety for a
specific purpose can be determined using methods well
known to those in the art.

10   The route of administration of a tumor homing
molecule will depend, in part, on the chemical structure
of the molecule. Peptides, for example, are not
particularly useful when administered orally because they
can be degraded in the digestive tract. However, methods

15   for chemically modifying peptides to render them less
susceptible to degradation by endogenous proteases or
more absorbable through the alimentary tract are well
known (see, for example, Blondelle et al., *supra*, 1995;
Ecker and Crooke, *supra*, 1995; Goodman and Ro, *supra*,

20   1995). Such modifications can be performed on peptides
identified by *in vivo* panning. In addition, methods for
preparing libraries of peptidomimetics, which can contain
D-amino acids, other non-naturally occurring amino acids,
or chemically modified amino acids; or can be organic

25   molecules that mimic the structure of peptide; or can be
peptoids such as vinylogous peptoids, are known in the
art and can be used to identify molecules that home to a
tumor and are stable for oral administration.

30   Tumor homing molecules obtained using the
methods disclosed herein also can be useful for
identifying a target molecule such as a cell surface
receptor or a ligand for a receptor, which is recognized
by the tumor homing peptide, or for substantially

35   isolating the target molecule. For example, a tumor
homing peptide can be linked to a solid support such as a
chromatography matrix. The linked peptide then can be

used for affinity chromatography by passing an
appropriately processed sample of a tumor over the column
in order to bind a particular target molecule. The
target molecule, which forms a complex with the tumor
5   homing molecule, then can be eluted from the column and
collected in a substantially isolated form. The
substantially isolated target molecule then can be
characterized using well known methods. A tumor homing
peptide also can be linked to a detectable moiety such as
10 a radionuclide, a fluorescent molecule, an enzyme or
biotin and can be used, for example, to screen a sample
in order to detect the presence of the target molecule in
a tumor or to follow the target molecule during various
isolation steps.
15

     It follows that, upon identifying the presence
of a target molecule in a tumor sample, the skilled
artisan readily can obtain the target molecule in a
substantially isolated form. For example, the sample
20 containing the target molecule can be passed over a
column containing attached thereto the relevant tumor
homing molecule, thereby providing a means to obtain the
target molecule in substantially isolated form. Thus,
the invention further provides a substantially isolated
25 target molecule, which specifically binds a tumor homing
molecule and which can be obtained using the methods
disclosed herein.

     The methods of the present invention were used
30 to identify tumor homing peptides, which can selectively
home to various tumors. It should be recognized that
cysteine residues were included in some peptides such
that cyclization of the peptides could be effected. In
fact, the peptides containing at least two cysteine
35 residues cyclize spontaneously. However, such cyclic
peptides also can be active when present in a linear form
(see, for example, Koivunen et al., supra, 1993) and, as

disclosed herein, a linear peptide, NGRAHA (SEQ ID
NO: 6), also was useful as tumor homing molecule
(Example VII; see, also, Table 1). Thus, in some cases
one or more cysteine residues in the peptides disclosed
5   herein or otherwise identified as tumor homing peptides
can be deleted without significantly affecting the tumor
homing activity of the peptide. Methods for determining
the necessity of a cysteine residue or of amino acid
residues N-terminal or C-terminal to a cysteine residue
10 for tumor homing activity of a peptide of the invention
are routine and well known in the art.

A tumor homing peptide is useful, for example,
for targeting a desired moiety to the selected tumor as
15 discussed above. In addition, a tumor homing peptide can
be used to identify the presence of a target molecule in
a sample. As used herein, the term "sample" is used in
its broadest sense to mean a cell, tissue, organ or
portion thereof, including a tumor, that is isolated from
20 the body. A sample can be, for example, a histologic
section or a specimen obtained by biopsy or cells that
are placed in or adapted to tissue culture. If desired,
a sample can be processed, for example, by
homogenization, which can be an initial step for
25 isolating the target molecule to which a tumor homing
molecule binds.

A tumor homing peptide such as a breast tumor
homing peptide can be used to identify the target
30 molecule expressed in a breast tumor. For example, a
breast tumor homing peptide can be attached to a matrix
such as a chromatography matrix to produce a peptide
affinity matrix. A homogenized sample of a breast tumor
can be applied to the peptide-affinity matrix under
35 conditions that allow specific binding of the target
molecule to the tumor homing peptide (see, for example,
Deutshcer, Meth. Enzymol., Guide to Protein Purification

(Academic Press, Inc., ed. M.P. Deutscher, 1990),
Vol. 182, which is incorporated herein by reference; see,
for example, pages 357-379). Unbound and nonspecifically
bound material can be removed and the specifically bound

5 breast tumor-derived target molecule can be isolated in
substantially purified form. The presence or absence of
the target molecule in normal breast tissue also can be
determined. Such an analysis can provide insight into
methods of treating the tumor.

10

     As disclosed herein, a target molecule, which
specifically binds a tumor homing molecule, can be
identified by contacting a sample of a tumor with such a
tumor homing molecule and identifying a target molecule

15 bound by the tumor homing molecule. In parallel, the
tumor homing molecule is contacted with a sample of a
nontumor tissue corresponding to the tumor. The presence
of the target molecule in the tumor sample can be
identified by determining that the tumor homing molecule

20 does not bind to a component of the corresponding
nontumor tissue sample. Thus, the invention provides
methods for identifying the presence of a target
molecule, which is expressed in a tumor and specifically
bound by a tumor homing molecule.

25

     Since numerous tumor homing peptides containing
the NGR motif have been identified, for example, a tumor
homing peptide comprising an NGR sequence can be used to
isolate the NGR receptor. Thus, an NGR tumor homing

30 peptide can be linked to a solid matrix and an
appropriately processed sample of a tumor, which
specifically binds the NGR peptide, can be passed over
the NGR peptide-matrix. The NGR receptor, which is the
target molecule for the NGR tumor homing peptide, then

35 can be obtained in a substantially isolated form. When
used in reference to a target molecule, the term
"substantially isolated" means that the target molecule

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comprises at least 30% of the total protein present,
although the target molecule can comprise at least 50% of
the total protein, or 80% of the total protein, or 90% or
95% of the total protein, or more. A method such as gel
5   electrophoresis and silver staining can be used to
determine the relative amount of a target molecule in a
sample, following a purification protocol, and,
therefore, can be used to identify a substantially
isolated target molecule.
10

        The skilled artisan will recognize that a
substantially isolated target molecule can be used as an
immunogen to obtain antibodies that specifically bind the
target molecule. As used herein, the term "antibody" is
15   used in its broadest sense to include polyclonal and
monoclonal antibodies, as well as antigen binding
fragments of such antibodies. With regard to an antibody
of the invention, which specifically binds a target
molecule targeted by a tumor homing molecule, the term
20   "antigen" means the target molecule polypeptide or
peptide portion thereof. An antibody or antigen binding
fragment of an antibody that binds a target molecule is
characterized by having specific binding activity for the
target molecule or a peptide portion thereof of at least
25   about 1 x 10^5 M^{-1}, preferably at least about 1 x 10^6 M^{-1},
and more preferably at least about 1 x 10^8 M^{-1}. Thus,
Fab, F(ab')$_2$, Fd and Fv fragments of the antibody, which
retain specific binding activity for a target molecule,
which is expressed by angiogenic vasculature, are
30   included within the definition of an antibody.

        In addition, the term "antibody" as used herein
includes naturally occurring antibodies as well as
non-naturally occurring antibodies, including, for
35   example, single chain antibodies, chimeric, bifunctional
and humanized antibodies, as well as antigen-binding
fragments thereof. Such non-naturally occurring

antibodies can be constructed using solid phase peptide
synthesis, can be produced recombinantly or can be
obtained, for example, by screening combinatorial
libraries consisting of variable heavy chains and
5   variable light chains as described by Huse et al.,
Science 246:1275-1281 (1989), which is incorporated
herein by reference. These and other methods of making,
for example, chimeric, humanized, CDR-grafted, single
chain, and bifunctional antibodies are well known to
10   those skilled in the art (Winter and Harris, Immunol.
Today 14:243-246 (1993); Ward et al., Nature 341:544-546
(1989); Hilyard et al., Protein Engineering: A practical
approach (IRL Press 1992); Borrabeck, Antibody
Engineering, 2d ed. (Oxford University Press 1995); each
15   of which is incorporated herein by reference; see, also,
Harlow and Lane, *supra*, 1988).

     Antibodies that specifically bind a target
molecule of the invention can be raised using as an
20   immunogen a substantially isolated target molecule, which
can be obtained as disclosed herein, or a peptide portion
of the target molecule, which can be obtained, for
example, by enzymatic degradation of the target molecule
and gel purification. A non-immunogenic peptide portion
25   of a target molecule can be made immunogenic by coupling
the hapten to a carrier molecule such as bovine serum
albumin (BSA) or keyhole limpet hemocyanin (KLH).
Various other carrier molecules and methods for coupling
a hapten to a carrier molecule are well known in the art
30   and described, for example, by Harlow and Lane, *supra*,
1988).

     Particularly useful antibodies of the invention
are those that bind to the tumor homing molecule binding
35   site on the target molecule, such antibodies being
readily identifiable by detecting competitive inhibition
of binding of the antibody and the particular tumor

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homing molecule that binds to the target molecule.
Conversely, antibodies that bind to an epitope of the
target molecule that is not involved in binding the tumor
homing molecule also are valuable, since such antibodies,

5 which, themselves, can be "tumor homing molecules," can
be bind to target molecules having another tumor homing
molecule bound thereto.

An antibody that specifically binds a target
10 molecule, for example, the NGR receptor, is useful for
determining the presence or level of the target molecule
in a tissue sample, which can be a lysate or a
histological section. The identification of the presence
or level of the target molecule in the sample can be made
15 using well known immunoassay and immunohistochemical
methods (Harlow and Lane, *supra*, 1988). An antibody
specific for a target molecule also can be used to
substantially isolate the target molecule from a sample.
In addition, an antibody of the invention can be used in
20 a screening assay to identify, for example,
peptidomimetics of a tumor homing molecule that bind to
the target molecule or as a tool for tumor targeting.

Upon obtaining a target molecule, which, due to
25 the nature of a tumor homing molecule, is expressed in
angiogenic vasculature, for example, the angiogenic
vasculature in a tumor, the naturally occurring ligand
for the target molecule, where it exists, can be
identified. Methods for identifying a ligand for such a
30 target molecule, which is akin to an "orphan receptor,"
are well known in the art and include, for example,
screening biological samples to identify the ligand. A
convenient screening assay to identify a natural ligand
for the target molecule can utilize the ability of a
35 putative natural ligand to competitively inhibit the
binding to the target molecule of a tumor homing molecule
that specifically binds the target molecule, for example,

the tumor homing peptide used to obtain the substantially
isolated target molecule.

A screening assay comprising a competitive
5   binding assay for the target molecule and, for example,
the natural ligand for the target molecule or a tumor
homing peptide that specifically binds the target
molecule, also provides a means to identify
peptidomimetics of a tumor homing molecule. As discussed
10 above, such peptidomimetics can provide advantages over
tumor homing peptides in that they can be small,
relatively stable for storage, conveniently produced in
suitable quantities, and capable of being administered
orally. A peptidomimetic of a tumor homing peptide can
15 be identified by screening libraries of peptidomimetics
in a competitive binding assay as described above.

The disclosed *in vivo* panning method can be
used to detect four different kinds of target molecules
20 in tumors. First, because tumor vasculature undergoes
active angiogenesis, target molecules that are
characteristic of angiogenic vasculature, in general, or
angiogenic tumor vasculature, in particular, can be
identified. Second, vascular target molecules that are
25 characteristic of the tissue of origin of the tumor can
be identified. Third, target molecules that are
expressed in the vasculature of a particular type of
tumor can be identified. Fourth, tumor stroma or tumor
cell target molecules can be identified due to the
30 fenestrated nature of tumor vasculature, which allows the
potential tumor homing molecules to leave the circulation
and contact the tumor parenchyma.

As further disclosed herein, some, but not all,
35 tumor homing molecules also can home to angiogenic
vasculature that is not contained within a tumor. For
example, tumor homing molecules containing either the RGD

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motif or the GSL motif specifically homed to retinal
neovasculature (Smith et al., _Invest. Ophthamol. Vis.
Sci._ 35:101-111 (1994), which is incorporated herein by
reference), whereas tumor homing peptides containing the
5   NGR motif did not accumulate substantially to this
angiogenic vasculature. Thus, the present invention also
provides peptides that home to nontumor angiogenic
vasculature. Furthermore, these results indicate that
tumor vasculature expressing target molecules that are
10   not substantially expressed by other kinds of angiogenic
vasculature. Thus, the present invention provides a
means to identify target molecules expressed specifically
by angiogenic vasculature present in a tumor, as well as
for target molecules expressed by angiogenic vasculature
15   not associated with a tumor. Methods as disclosed herein
can be used to distinguish such homing peptides and to
isolate the various target molecules.

As an alternative to using a tumor sample to
20   obtain the target molecule, extracts of cultured tumor
cells or endothelial cells, depending on which cell type
expresses the target molecule, can be used as the
starting material in order to enhance the concentration
of the target molecule in the sample. It is recognized,
25   however, that the characteristics of such cells can
change upon adaptation to tissue culture. Thus, care
must be exercised if such a preselection step is
attempted. The presence of the target molecule can be
established, for example, by using phage binding and cell
30   attachment assays (see, for example, Barry et al., _supra_,
1996).

A cell line expressing a particular target
molecule can be identified and surface iodination of the
35   cells can be used to label the target molecule. The
cells then can be extracted, for example, with
octylglucoside and the extract can be fractionated by

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affinity chromatography using a tumor homing peptide (see
Tables 1 and 2) coupled to a matrix such as SEPHAROSE
(see Hermanson, *supra*, 1996). The purified target
molecule can be microsequenced and antibodies can be
5   prepared. If desired, oligonucleotide probes can be
prepared and used to isolate cDNA clones encoding the
target molecule. Alternatively, an anti-target molecule
antibody can be used to isolate a cDNA clone from an
expression library (see Argraves et al., *J. Cell Biol.*
10 105:1183-1190 (1987), which is incorporated herein by
reference).

As an alternative to isolating the target
molecule, a nucleic acid encoding the target molecule can
15 be isolated using a mammalian cell expression cloning
system such as the COS cell system. An appropriate
library can be prepared, for example, using mRNA from
primary tumor cells. The nucleic acids can be cloned
into the pcDNAIII vector (Invitrogen), for example.
20 Cells expressing a cDNA for the target molecule can be
selected by binding to the tumor homing peptide.
Purified phage can be used as the carrier of the peptide
and can be attached to magnetic beads coated, for
example, with anti-M13 antibodies (Pharmacia Biotech;
25 Piscataway NJ). Cells that bind to the peptide coating
can be recovered using a magnet and the plasmids can be
isolated. The recovered plasmid preparations then can be
divided into pools and examined in COS cell
transfections. The procedure can be repeated until
30 single plasmids are obtained that can confer upon the COS
cells the ability to bind the tumor homing peptide.

The following examples are intended to
illustrate but not limit the present invention.
35

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EXAMPLE I

IN VIVO PANNING

This example demonstrates methods for preparing

5 a phage library and screening the library using *in vivo* panning to identify phage expressing peptides that home to a tumor.

A. Preparation of phage libraries:

10

Phage display libraries were constructed using the fuse 5 vector as described by Koivunen et al. (*supra*, 1995; Koivunen et al., *supra*, 1994b). Libraries encoding peptides designated CX$_5$C (SEQ ID NO: 9), CX$_6$C (SEQ ID

15 NO: 10), CX$_7$C (SEQ ID NO: 11) and CX$_3$CX$_3$CX$_3$C (SEQ ID NO: 12) were prepared, where "C" indicates cysteine and "X$_N$" indicates the given number of individually selected amino acids. These libraries can display cyclic peptides when at least two cysteine residues are present in the

20 peptide. In addition, a library that did not contain defined cysteine residues also was constructed. Such a library results in the production primarily of linear peptides, although cyclic peptides also can occur due to random probability.

25

A biased library based on the sequence CXXXNGRXX (SEQ ID NO: 13) also was constructed. Furthermore, in some cases the CXXXNGRXX (SEQ ID NO: 13) library was further biased by in the incorporation of

30 cysteine residues flanking the NGR sequence, i.e., CXXCNGRCX (SEQ ID NO: 14; see Table 1).

The libraries containing the defined cysteine residues were generated using oligonucleotides

35 constructed such that "C" was encoded by the codon TGT and "X$_N$" was encoded by NNK, where "N" is equal molar mixtures of A, C, G and T, and where "K" is equal molar

71

mixtures of G and T. Thus, the peptide represented by
CX_5C (SEQ ID NO: 9) can be represented by an
oligonucleotide having the sequence $TGT(NNK)_5TGT$ (SEQ ID
NO: 14). Oligonucleotides were made double stranded by 3
5   cycles of PCR amplification, purified and ligated to the
nucleic acid encoding the gene III protein in the fuse 5
vector such that, upon expression, the peptide is present
as a fusion protein at the N-terminus of the gene III
protein.
10

        The vectors were transfected by electroporation
into MC1061 cells. Bacteria were cultured for 24 hr in
the presence of 20 μg/ml tetracycline, then phage were
collected from the supernatant by precipitation twice
15 using polyethylene glycol. Each library contained about
5×10^9 to 5×10^{14} transducing units (TU; individual
recombinant phage).

        B.   In vivo panning of phage:
20

        Tumors were transplanted into mice as described
in Examples II and III, below. A mixture of phage
libraries containing 1×10^9 to 1×10^{14} TU was diluted in
200 μl DMEM and injected into the tail vein of
25 anesthetized mice (AVERTIN (0.015 ml/g); see U.S. Patent
No. 5,622,699; Pasqualini and Ruoslahti, supra, 1996).
After 1-4 minutes, mice were snap frozen in liquid
nitrogen. To recover the phage, carcasses were partially
thawed at room temperature for 1 hr, tumors and control
30 organs were collected and weighed, then were ground in
1 ml DMEM-PI (DMEM containing protease inhibitors (PI);
phenylmethylsulfonyl fluoride (PMSF; 1 mM), aprotinin
(20 μg/ml), leupeptin (1 μg/ml)).

35   Alternatively, following introduction of a
library into a mouse, circulation of the library is
terminated by perfusion through the heart. Briefly, mice

72

were anesthetized with AVERTIN, then the heart was
exposed and a 0.4 mm needle connected through a 0.5 mm
cannula to a 10 cc syringe was inserted into the left
ventricle. An incision was made on the right atrium and
5   5 to 10 ml of DMEM was slowly administered, perfusing the
whole body over about a 5 to 10 min period. Efficiency
of the perfusion was monitored directly by histologic
analysis.

10   Tumor and organ samples were washed 3 times
with ice cold DMEM-PI containing 1% bovine serum albumin
(BSA), then directly incubated with 1 ml K91-kan bacteria
for 1 hr. Ten ml NZY medium containing 0.2 μg/ml
tetracycline (NZY/tet) was added to the bacterial
15   culture, the mixture was incubated in a 37°C shaker for
1 hr, then 10 μl or 100 μl aliquots were plated in agar
plates containing 12.5 μg/ml tetracycline (tet/agar).

            Individual colonies containing phage recovered
20   from a tumor were grown for 16 hr in 5 ml NZY/tet. The
bacterial cultures obtained from the individual colonies
were pooled and the phage were purified and re-injected
into mice as described above for a second round of *in
vivo* panning. In general, a third round of panning also
25   was performed. Phage DNA was purified from individual
bacterial colonies obtained from the final round of *in
vivo* panning and the DNA sequences encoding the peptides
expressed by selected phage were determined (see Koivunen
et al., *supra*, 1994b).
30

EXAMPLE II

IDENTIFICATION OF TUMOR HOMING PEPTIDES
BY *IN VIVO* PANNING AGAINST A BREAST TUMOR

35   This example demonstrates that *in vivo* panning
can be performed against a breast tumor to identify tumor
homing peptides that home to various tumors.

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Human 435 breast carcinoma cells (Price et al.,
Cancer Res. 50:717-721 (1990)) were inoculated into the
mammary fat pad of nude mice. When the tumors attained a
diameter of about 1 cm, either a phage targeting
5 experiment was performed, in which phage expressing a
specific peptide were administered to the tumor bearing
mouse, or *in vivo* panning was performed.

The breast tumor bearing mice were injected
10 with 1 x 10^9 phage expressing a library of CX$_3$CX$_3$CX$_3$C (SEQ
ID NO: 12) peptides, where X$_3$ indicates three groups of
independently selected, random amino acids. The phage
were allowed to circulate for 4 min, then the mice were
anesthetized, snap frozen in liquid nitrogen while under
15 anesthesia, and the tumor was removed. Phage were
isolated from the tumor and subjected to two additional
rounds of *in vivo* panning.

Following the third round of panning, phage
20 were quantitated and the peptide sequences expressed by
the cloned phage were determined. The cloned
phage expressed various different peptides, including
those shown in Table 1. Similarly, CX$_7$C (SEQ ID NO: 11)
and CX$_5$C (SEQ ID NO: 9) libraries were screened and breast
25 tumor homing peptides were identified (Table 1). These
results demonstrate that *in vivo* panning against a breast
tumor can identify tumor homing molecules.

EXAMPLE III
30 *IN VIVO TARGETING OF A PHAGE EXPRESSING AN*
AN RGD PEPTIDE TO A TUMOR

Human 435 breast carcinoma cells were
inoculated into the mammary fat pad of nude mice. When
35 the tumors attained a diameter of about 1 cm, phage
expressing a specific RGD-containing peptide were
administered to the tumor bearing mouse. Similar results

TABLE 1

PEPTIDES FROM PHAGE RECOVERED FROM HUMAN BREAST CANCER

CGRECPRLCQSSC (2*)	CNGRCVSGCAGRC (3)	
CGEACGGQCALPC (20)	IWSGYGVYW (21)	
PSCAYMCIT (22)	WESLYFPRE (23)	
SKVLYYNWE (24)	CGLMCQGACFDVC (25)	
CERACRNLCREGC (26)	CPRGCLAVCVSQC (27)	
CKVCNGRCCG (28)	CEMCNGRCMG (29)	CPLCNGRCAL (30)
CPTCNGRCVR (31)	CGVCNGRCGL (32)	CEQCNGRCGQ (33)
CRNCNGRCEG (34)	CVLCNGRCWS (35)	CVTCNGRCRV (36)
CTECNGRCQL (37)	CRTCNGRCLE (38)	CETCNGRCVG (39)
CAVCNGRCGF (40)	CRDLNGRKVM (41)	CSCCNGRCGD (42)
CWGCNGRCRM (43)	CPLCNGRCAR (44)	CKSCNGRCLA (45)
CVPCNGRCHE (46)	CQSCNGRCVR (47)	CRTCNGRCQV (48)
CVQCNGRCAL (49)	CRCCNGRCSP (50)	CASNNGRVVL (51)
CGRCNGRCLL (52)	CWLCNGRCGR (53)	CSKCNGRCGH (54)
CVWCNGRCGL (55)	CIRCNGRCSV (56)	CGECNGRCVE (57)
CEGVNGRRLR (58)	CLSCNGRCPS (59)	CEVCNGRCAL (60)
CGSLVRC (5)	GRSQMQI (61)	HHTRFVS (62)
SKGLRHR (63)	VASVSVA (64)	WRVLAAF (65)
KMGPKVW (66)	IFSGSRE (67)	SPGSWTW (68)
NPRWFWD (69)	GRWYKWA (70)	IKARASP (71)
SGWCYRC (72)	ALVGLMR (73)	LWAEMTG (74)
CWSGVDC (75)	DTLRLRI (76)	SKSSGVS (77)
IVADYQR (78)	VWRTGHL (79)	VVDRFPD (80)
LSMFTRP (81)	GLPVKWS (82)	IMYPGWL (83)
CVMVRDGDC (84)	CVRIRPC (85)	CQLAAVC (86)
CGVGSSC (87)	CVSGPRC (88)	CGLSDSC (89)
CGEGHPC (90)	CYTADPC (91)	CELSLISKC (92)
CPEHRSLVC (93)	CLVVHEAAC (94)	CYVELHC (95)
CWRKFYC (96)	CFWPNRC (97)	CYSYFLAC (98)
CPRGSRC (99)	CRLGIAC (100)	CDDSWKC (101)
CAQLLQVSC (102)	CYPADPC (103)	CKALSQAC (104)
CTDYVRC (105)	CGETMRC (106)	

* - numbers in parentheses indicate SEQ ID NO:.

75

to those discussed below also were obtained with nude
mice bearing tumors formed by implantation of human
melanoma C8161 cells or by implantation of mouse B16
5   melanoma cells.

     1 x 10^9 phage expressing the RGD-containing
peptide, CDCRGDCFC (SEQ ID NO: 1; see, Koivunen et al.,
supra, 1995) or control (insertless) phage were injected
10   intravenously (iv) into the mice and allowed to circulate
for 4 min. The mice then were snap frozen or perfused
through the heart while under anesthesia, and various
organs, including tumor, brain and kidney, were removed
and the phage present in the organs was quantitated (see
15   U.S. Patent No. 5,622,699; Pasqualini and Ruoslahti,
supra, 1996).

     Approximately 2-3 times more phage expressing
the CDCRGDCFC (SEQ ID NO: 1) peptide were detected in the
20   breast tumor as compared to brain and kidney, indicating
the CDCRGDCFC (SEQ ID NO: 1; RGD phage) peptide resulted
in selective homing of the phage to the breast tumor. In
a parallel study, unselected phage, which express
various, diverse peptides, were injected into tumor-
25   bearing mice and various organs were examined for the
presence of phage. Far more phage were present in kidney
and, to a lesser extent, brain, as compared to the tumor.
Thus, the 80-fold more RGD-expressing phage than
unselected phage concentrated in the tumor. These
30   results indicate that phage expressing the RGD-containing
peptide home to a tumor, possibly due to the expression
of the $\alpha_v\beta_3$ integrin on blood vessels forming in the
tumor.

35   Specificity of the breast tumor homing peptide
was demonstrated by competition experiments, in which
coinjection of 500 µg free peptide, ACDCRGDCFCG (SEQ ID

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NO: 16; see Pasqualini et al., *supra*, 1997) with the
phage expressing the tumor homing peptide reduced the
amount of phage in the tumor by about tenfold, whereas
coinjection with the inactive control peptide, GRGESP

5   (SEQ ID NO: 17) essentially had no effect. These results
demonstrate that phage displaying a peptide that can bind
to an integrin expressed on angiogenic vasculature can
selectively home *in vivo* to an organ or tissue such as a
tumor containing such vasculature.

10

EXAMPLE IV
IMMUNOHISTOLOGIC ANALYSIS OF TUMOR HOMING PEPTIDES

This example provides a method of identifying

15   the localization of tumor homing molecules by
immunohistologic examination.

Localization of phage expressing a tumor homing
peptide was identified by immunochemical methods in

20   histologic sections obtained either 5 min or 24 hr after
administration of phage expressing a tumor homing peptide
("peptide-phage") to a tumor bearing mouse (Figure 1).
For samples obtained 5 min following administration of
the peptide-phage, mice were perfused with DMEM and

25   various organs, including the tumor, were removed and
fixed in Bouin's solution. For samples obtained at
24 hr, no peptide-phage remains in the circulation and,
therefore, perfusion was not required. Histologic
sections were prepared and reacted with anti-M13 (phage)

30   antibodies (Pharmacia Biotech; see U.S. Patent
No. 5,622,699; Pasqualini and Ruoslahti, *supra*, 1996).
Visualization of the bound anti-M13 antibody was
performed using a peroxidase-conjugated second antibody
(Sigma; St. Louis MO) according to the manufacturer's

35   instructions.

As discussed in Example III, phage expressing the tumor homing peptide, CDCRGDCFC (SEQ ID NO: 1; "RGD phage), were administered intravenously to mice bearing the breast tumor. In addition, the RGD phage were

5   administered to mice bearing a mouse melanoma or a human Kaposi's sarcoma. Circulation of the phage was terminated and mice were sacrificed as described above and samples of the tumor and of skin adjacent to the tumor, brain, kidney, lung and liver were collected.

10   Immunohistochemical staining for the phage showed accumulation of the RGD phage in the blood vessels present in the breast tumor as well as in the melanoma and the Kaposi's sarcoma, whereas little or no staining was observed in the control organs.

15

Similar experiments were performed using phage expressing the tumor homing peptide, CNGRCVSGCAGRC (SEQ ID NO: 3; "NGR phage"), which was identified by *in vivo* panning against a tumor formed by the MDA-MB-435

20   breast carcinoma. In these experiments, NGR phage or control phage, which do not express a peptide, were administered to mice bearing tumors formed by the MDA-MB-435 breast carcinoma or by a human SLK Kaposi's sarcoma xenograft, then the mice were sacrificed as

25   described above and tumors were collected as well as control organs, including brain, lymph node, kidney, pancreas, uterus, mammary fat pad, lung, intestine, skin, skeletal muscle, heart and epithelium of the renal calices, bladder and ureter (see Figure 1). Histological

30   samples were prepared and examined by immunostaining as described above.

In samples obtained from mice sacrificed 4 min after administration of the NGR phage, immunostaining of

35   the vasculature of both the breast tumor (Figure 1E) and the Kaposi's sarcoma (Figure 1H) was observed. Very little or no staining was observed in the endothelium of

the these tumors in mice administered an insertless
control phage (Figures 1G and 1J, respectively). In the
samples obtained from mice sacrificed 24 hr after
administration of the NGR phage, staining of the tumor
5   samples appeared to have spread outside of the vessels,
into the breast tumor parenchyma (Figures 1B and 1F) and
the Kaposi's sarcoma parenchyma (Figures 1D and 1I).
Again, little or no staining was observed in samples
prepared from these tumors in mice administered the
10   insertless control phage (Figures A and C, respectively).
In addition, little or no staining was observed in
various control organs in mice administered the NGR phage
(Figures 1K to 1V).

15   In other experiments, similar results were
obtained following administration of phage expressing the
NGR tumor homing peptides, NGRAHA (SEQ ID NO: 6) or
CVLNGRMEC (SEQ ID NO: 7), to tumor bearing mice. Also,
as discussed below, similar results were obtained using
20   phage expressing the GSL tumor homing peptide, CLSGSLSC
(SEQ ID NO: 4), which was identified by *in vivo* panning
of a melanoma (see Example V, below).

           These results demonstrate that tumor homing
25   peptides selectively home to tumors, particularly to the
vasculature in the tumors and that tumor homing peptides
identified, for example, by *in vivo* panning against a
breast carcinoma also selectively home to other tumors,
including Kaposi's sarcoma and melanoma. In addition,
30   these results demonstrate that immunohistochemical
analysis provides a convenient assay for identifying the
localization of phage expressing tumor homing peptides.

EXAMPLE V

IDENTIFICATION OF TUMOR HOMING PEPTIDES
BY IN VIVO PANNING AGAINST A MELANOMA TUMOR

5   The general applicability of the *in vivo* panning method to identify tumor homing peptides was examined by performing *in vivo* panning against an implanted mouse melanoma tumor.

10   Mice bearing a melanoma were produced by implantation of B16B15b mouse melanoma cells, which produce highly vascularized tumors. B16B15b mouse melanoma cells were injected subcutaneously into the mammary fat pad of nude mice (2 months old) and tumors 15 were allowed to grow until the diameter was about 1 cm. *In vivo* panning was performed as disclosed above. Approximately 1×10^{12} transducing units of phage expressing the CX_5C (SEQ ID NO: 9), CX_6C (SEQ ID NO: 10) or CX_7C (SEQ ID NO: 11) library were injected, iv, and 20 allowed to circulate for 4 min. Mice then were snap frozen in liquid nitrogen or perfused through the heart while under anesthesia, tumor tissue and brain (control organ) were removed, and phage were isolated as described above. Three rounds of *in vivo* panning were performed.
25

      The amino acid sequences were determined for the inserts in 89 cloned phage recovered from the B16B15b tumors. The peptides expressed by these phage were represented by two predominant sequences, CLSGSLSC (SEQ 30 ID NO: 4; 52% of the clones sequenced) and WGTGLC (SEQ ID NO: 18; 25% of the clones; see Table 2). Reinfection of phage expressing one of the selected peptides resulted in approximately three-fold enrichment of phage homing to the tumor relative to brain.

TABLE 2

PEPTIDES FROM PHAGE RECOVERED FROM MOUSE B16B15b MELANOMA

CLSGSLSC (4*)	GICKDDWCQ (107)	TSCDPSLCE (108)
KGCGTRQCW (109)	YRCREVLCQ (110)	CWGTGLC (111)
WSCADRTCM (112)	AGCRLKSCA (113)	SRCKTGLCQ (114)
PICEVSRCW (115)	WTCRASWCS (116)	GRCLLMQCR (117)
TECDMSRCM (118)	ARCRVDPCV (119)	CIEGVLGGC (120)
CSVANSC (121)	CSSTMRC (122)	SIDSTTF (123)
GPSRVGG (124)	WWSGLEA (125)	LGTDVRQ (126)
LVGVRLL (127)	GRPGDIW (128)	TVWNPVG (129)
GLLLVVP (130)	FAATSAE (131)	WCCRQFN (132)
VGFGKAL (133)	DSSLRLP (134)	KLWCAMS (135)
SLVSFLG (136)	GSFAFLV (137)	IASVRWA (138)
TWGHLRA (139)	QYREGLV (140)	QSADRSV (141)
YMFWTSR (142)	LVRRWYL (143)	TARGSSR (144)
TTREKNL (145)	PKWLLFS (146)	LRTNVVH (147)
AVMGLAA (148)	VRNSLRN (149)	

* – numbers in parentheses indicate SEQ ID NO:.

Localization of the phage expressing a tumor homing peptide in the mouse organs also was examined by immunohistochemical staining of the tumor and various other tissues (see Example IV). In these experiments, 1 x 10^9 pfu of a control (insertless) phage or a phage expressing the tumor homing peptide, CLSGSLSC (SEQ ID NO: 4), were injected, iv, into tumor bearing mice and allowed to circulate for 4 min.

Immunostaining was evident in the melanoma obtained from a mouse injected with phage expressing the CLSGSLSC (SEQ ID NO: 4) tumor homing peptide. Staining

of the melanoma generally was localized to the blood
vessels within the tumor, although some staining also was
present in the tumor parenchyma. Essentially no staining
was observed in a tumor obtained from a mouse injected

5 with the insertless control phage or in skin or in kidney
samples obtained from mice injected with either phage.
However, immunostaining was detected in the liver
sinusoids and in spleen, indicating that phage can be
trapped nonspecifically in organs containing RES.

10

Using similar methods, *in vivo* panning was
performed in mice bearing a SLK human Kaposi's sarcoma.
Tumor homing peptides were identified and are disclosed
in Table 3. Together, these results demonstrate that the

15 *in vivo* panning method is a generally applicable method
for screening a phage library to identify phage
expressing tumor homing peptides.

EXAMPLE VI

20 PREPARATION AND CHARACTERIZATION OF
TUMOR HOMING PEPTIDE/DOXORUBICIN CONJUGATES

This example provides methods for conjugating a
moiety such as the chemotherapeutic agent, doxorubicin,

25 to a tumor homing peptide and for characterizing the
conjugation reaction.

The peptides CDCRGDCFC (SEQ ID NO: 1; Koivunen
et al., *supra*, 1995; Pasqualini et al., *supra*, 1997),

30 CNGRC (SEQ ID NO: 8), CGSLVRC (SEQ ID NO: 5) and
GACVFSIAHECGA (SEQ ID NO: 19) were synthesized, cyclized
under high dilution and purified to homogeneity by HPLC.
Conjugation of the peptides to doxorubicin (Aldrich;
Milwaukee WI) was performed using 1-ethyl-3-

35 (3-dimethylaminopropyl)carbodiimide hydrochloride (EDC;
Sigma; St. Louis MO) and N-hydroxysuccinimide (NHS;

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TABLE 3

PEPTIDES FROM PHAGE RECOVERED FROM HUMAN KAPOSI'S SARCOMA

TDCTPSRCT (150*)	SWCQFEKCL (151)	VPCRFKQCW (152)
CTAMRNTDC (153)	CRESLKNC (154)	CMEMGVKC (155)
VTCRSLMCQ (156)	CNNVGSYC (157)	CGTRVDHC (158)
CISLDRSC (159)	CAMVSMED (160)	CYLGVSNC (161)
CYLVNVDC (162)	CIRSAVSC (163)	LVCLPPSCE (164)
RHCFSQWCS (165)	FYCPGVGCR (166)	ISCAVDACL (167)
EACEMAGCL (168)	PRCESQLCP (169)	RSCIKHQCP (170)
QWCSRRWCT (171)	MFCRMRSCD (172)	GICKDLWCQ (173)
NACESAICG (174)	APCGLLACI (175)	NRCRGVSCT (176)
FPCEGKKCL (177)	ADCRQKPCL (178)	FGCVMASCR (179)
AGCINGLCG (180)	RSCAEPWCY (181)	DTCRALRCN (182)
KGCGTRQCW (109)	GRCVDGGCT (183)	YRCIARECE (184)
KRCSSSLCA (185)	ICLLAHCA (186)	QACPMLLCM (187)
LDCLSELCS (188)	AGCRVESC (189)	HTCLVALCA (190)
IYCPGQECE (191)	RLCSLYGCV (192)	RKCEVPGCQ (193)
EDCTSRFCS (194)	LECVVDSCR (195)	EICVDGLCV (196)
RWCREKSCW (197)	FRCLERVCT (198)	RPCGDQACE (199)
CNKTDGDEGVTC (15)		

* - numbers in parentheses indicate SEQ ID NO:.

Sigma) as described (Bauminger and Wilchek, *supra*, 1980; Harlow and Lane, *supra*, 1988; Hurwitz et al., *supra*, 1975). Unreacted doxorubicin and peptide were removed from the doxorubicin/peptide conjugates by SEPHADEX G25 column chromatography using phosphate buffered saline. The conjugates were lyophilized for storage and were resuspended in sterile water prior to use.

●   ●

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HPLC, capillary electrophoresis and NMR
analyses were performed to characterize the conjugates.
HPLC-fluorescence was performed using an INTERSIL ODS-2
column (4.6 x 150 mm) and a mobile phase composed of
5   0.08% triethanolamine/0.02% phosphoric acid (85%)/27%
acetonitrile at 1 ml/min. Fluorescence detection was
performed with excitation at 490 nm and emission at
560 nm wavelength and the retention time (RT) and the
area under the curves (AUC) for doxorubicin (dox) and for
10 the major peaks was determined. Each of the conjugates
has a unique retention time, depending on the peptide, as
follows: dox/CDCRGDCFC (SEQ ID NO: 1), RT 7.4 min, AUC
26%; dox/CNGRC (SEQ ID NO: 8), RT 4.7 min, AUC 56%; and
dox/GACVFSIAHECGA (SEQ ID NO: 19), RT 7.7 min, AUC 43%.
15 In comparison, the retention time of doxorubicin is 10.6
min and, in the various reactions, the AUC was about 5%.

        Capillary electrophoresis (CE; Liu et al.,
supra, 1996) was performed in uncoated fused-silica
20 capillaries with 75 μm internal diameter and an effective
separation length of 50 cm. The CE detection system was
equipped with an UV absorbance detector and an argon
laser emitting at 488 nm. The laser beam is transmitted
via a fiber optic cable to the detector and illuminates
25 the capillary window and the fluorescence signal is
collected through an emission filter. Conjugation of
doxorubicin to the peptides changed the electrophoretic
characteristics of each of the conjugates, indicating
that this method can be used as a fast screening method
30 to identify progress of the conjugation reaction.

        One dimensional NMR analysis of the
doxorubicin/CNGRC conjugate revealed no evidence of
resonances arising from free doxorubicin. Two dimension
35 NMR analysis can allow a determination of the precise
molecular structure of the doxorubicin-peptide species.

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These results demonstrate that a moiety such as the cancer chemotherapeutic agent, doxorubicin, can be efficiently linked to tumor homing peptides of the invention to produce doxorubicin/tumor homing peptide
5 conjugates.

EXAMPLE VII

TUMOR THERAPY USING

DOXORUBICIN/TUMOR HOMING PEPTIDE CONJUGATES
10

This example demonstrates that doxorubicin/tumor homing peptide conjugates provide a therapeutic advantage over the use of doxorubicin, alone, for treating tumors.
15

Doxorubicin concentration of the conjugates (see Example V) was determined by measuring the optical absorbance of the solution at 490 nm in a standard spectrophotometer; this wavelength detects only the
20 doxorubicin, not the peptides. A calibration curve for doxorubicin was generated and used to calculate the concentration prior to use. Conjugation of doxorubicin to the various peptides did not affect this curve. This procedure ensures that each of the administered
25 conjugates contained the same amounts of doxorubicin equivalent.

In addition, the viability of tumor cells obtained from tumors of mice treated with a tumor homing
30 peptide/doxorubicin conjugate was compared to that of tumors from mice treated with free doxorubicin. In these experiments, breast tumor bearing mice were size matched with regard to the tumors, then treated intravenously with 30 µg equivalent of doxorubicin/CDCRGDCFC (SEQ ID
35 NO: 1) or of free doxorubicin. Five days after treatment, the mice were euthanized and the tumors were removed. The tumor pairs were weighed and ground and the

cell suspensions were plated (2 g tumor tissue per 150 mm plate).

Cell numbers were determined at 24 hours and 7 days after plating. Viability of tumor cells from the tumors of mice receiving the doxorubicin/CDCRGDCFC (SEQ ID NO: 1) conjugate was about 3 fold less than cells from tumors of mice treated with the free doxorubicin. These results demonstrate that administration to a tumor bearing mouse of a conjugate comprising a chemotherapeutic agent linked to a tumor homing molecule is more efficacious than administration of the agent, alone, in reducing the viability of tumor cells.

A. *In vitro characterization of cytotoxicity:*

MDA-MB-435 human breast carcinoma cells were plated at 1×10^5 cells/well in 96 well plates. Cells were incubated with increasing amounts of doxorubicin, the doxorubicin/CDCRGDCFC (SEQ ID NO: 1) conjugate, or the doxorubicin/GACVFSIAHECGA (SEQ ID NO: 19; control) conjugate (0.1 to 10 μg/well of doxorubicin-equivalent) for either 30 min or overnight. Following incubation, the agents were removed by extensive washing with PBS, then fresh medium added and incubation was continued. The number of surviving cells was determined at 24 hours with crystal violet staining (see Koivunen et al., *supra,* 1994).

In cells exposed to free doxorubicin, the doxorubicin/CDCRGDCFC (SEQ ID NO: 1) conjugate, or the doxorubicin/GACVFSIAHECGA (SEQ ID NO: 19) for 30 min, cell death was present only in the cultures treated with the doxorubicin/CDCRGDCFC (SEQ ID NO: 1) conjugate. However, if the agents were not removed after 30 min, the cells were killed by all of the treatments after 24 hr. These results indicate that enhanced cellular uptake

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occurs for the doxorubicin/CDCRGDCFC (SEQ ID NO: 1)
conjugate.

B.   *In vivo characterization of doxorubicin/tumor homing*
5   *peptide conjugates:*

        Female 2-month old Balb c nu/nu mice (Harlan
Sprague Dawley; San Diego CA) were used for these studies
and were cared for according to the Burnham Institute
10   animal facility guidelines. MDA-MB-435 breast carcinoma
cells (Price et al., Cancer Res. 50:717-721 (1993)) were
injected in the mammary fat pad of the nude mice and
tumor growth was monitored (Pasqualini et al., *supra*,
1997). Tumors were allowed to grow to a size of about
15   1 cm^3 (about 5% of the mouse's body weight) before
starting the treatment experiments, except for the
toxicity experiments as discussed below.

        Weekly doxorubicin/peptide conjugate or control
20   treatments (5 μg/mouse/week of doxorubicin-equivalent)
were administered intravenously. In some experiments, as
indicated, a dose of 30 μg/mouse was administered every
3 weeks. Treatment with doxorubicin, alone, is referred
to as "dox control" and treatment with doxorubicin
25   conjugated to the non-tumor homing control peptide,
GACVFSIAHECGA (SEQ ID NO: 19), is referred to as
"conjugate control." The results obtained in the dox
control groups as compared to the conjugate control
groups were not significantly different. As an
30   additional control, in some experiments the tumor homing
peptide was mixed with doxorubicin, without linking, and
the mixture was administered to tumor bearing mice. Such
treatment produced results that were not statistically
different from those obtained with the above described
35   dox controls.

●   ●

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Mice were anesthetized with a tribromoethanol-
based anesthetic mixture (AVERTIN; Papaioannou and Fox,
Lab. Anim. 43:189-192 (1993)) before each treatment.
Anesthetization facilitated the tail vein injections
5   (final volume, 200 μl) and allowed precise serial three
dimensional tumor size measurements. Tumor volume
calculations were based on the equation for the volume of
an ovaloid: V=4/3(πabc), where a, b, and c are 1/2 of
the measured diameters in each of the three dimensions.
10

At necropsy, MDA-MB-435 tumor-bearing mice
treated with the doxorubicin/CDCRGDCFC (SEQ ID NO: 1)
conjugate had significantly smaller tumors (t test,
p=0.02), less spread to regional lymph nodes (t test,
15   p<0.0001), a lower incidence of pulmonary metastasis and
fewer metastatic lesions (t test, p<0.0001) than the dox
control treated mice. All of the mice treated with the
doxorubicin/CDCRGDCFC (SEQ ID NO: 1) conjugate survived
beyond the time when the dox control and conjugate
20   control mice had died (Log-Rank test, p<0.0001; Wilcoxon
test, p=0.0007). Essentially the same results were
obtained in five separate experiments. These results
indicate that a doxorubicin/tumor homing peptide provides
a therapeutic advantage over doxorubicin, alone, in
25   reducing the growth of a primary tumor and preventing
metastasis of the tumor.

Gross and histopathologic examination was
performed on the mice. Many of the tumors in the mice
30   treated with 5 μg doxorubicin equivalent of
doxorubicin/CDCRGDCFC (SEQ ID NO: 1) presented marked
skin ulceration and tumor necrosis, whereas no such signs
were observed in dox control group or conjugate control
group. Histopathological analysis disclosed a pronounced
35   destruction of the vasculature in the tumors treated with
doxorubicin/CDCRGDCFC (SEQ ID NO: 1) conjugate (see

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Figures 2D to 2F) as compared to the dox control group
(Figures 2A to 2C).

5   In a dose escalation experiment, tumor bearing
mice were treated with the doxorubicin/CDCRGDCFC (SEQ ID
NO: 1) at 30 μg/mouse every three weeks for three cycles
and were observed, without further treatment, for an
extended period of time. The doxorubicin/CDCRGDCFC (SEQ
ID NO: 1) treated mice all remained alive more than
10 6 months after the dox control and conjugate control mice
had died. The results indicate that treatment with a
doxorubicin/tumor homing peptide conjugate can have a
curative effect.

15   Acute toxicity studies also were performed. In
these experiments, mice bearing extremely large tumors
(about 25% of body weight) were treated with 200 μg/mouse
doxorubicin or doxorubicin/CDCRGDCFC (SEQ ID NO: 1)
conjugate. All of the mice treated with the
20 doxorubicin/CDCRGDCFC (SEQ ID NO: 1) conjugate survived
for longer than one week, whereas all of the dox control
mice had died within 48 hr of treatment. These results
suggest that accumulation of the doxorubicin/CDCRGDCFC
(SEQ ID NO: 1) conjugate in the large tumors reduced the
25 circulating level of the conjugated doxorubicin, thus
reducing its toxicity.

        Similar results were obtained using the
doxorubicin/CNGRC (SEQ ID NO: 8) conjugate. In each of
30 three series of experiments, tumors in the mice treated
with doxorubicin/CNGRC (SEQ ID NO: 8) were significantly
smaller than tumor is the dox control and conjugate
control groups. Treatment with the doxorubicin/CNGRC
(SEQ ID NO: 8) conjugate almost completely suppressed
35 tumor growth, whereas free doxorubicin and doxorubicin
conjugated to the control peptide had essentially no
effect on tumor growth relative to treatment with the

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vehicle, alone. A marked effect on survival also was observed and some of the doxorubicin/CNGRC (SEQ ID NO: 8) treated animals survived for extended periods of time (Log-Rank test, p=0.0064; Wilcoxon test, p=0.0343). In
5   addition, the doxorubicin/CNGRC (SEQ ID NO: 8) conjugate was less toxic than free doxorubicin. These results confirm that conjugates comprising a chemotherapeutic agent and a tumor homing molecule provide a therapeutic advantage in treating cancer.
10

Although the invention has been described with reference to the disclosed examples, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly,
15 the invention is limited only by the following claims.

We claim:

1. A conjugate, comprising a tumor homing
peptide linked to a moiety, said tumor homing peptide
5 obtained by *in vivo* panning, comprising the steps of:

        a) administering to a first subject
        having a tumor a library of diverse peptides;

10   b) collecting a sample of the tumor;

        c) identifying a peptide that homes to
        said tumor;

15   d) collecting a sample of normal tissue
        corresponding to said tumor; and

        e) determining that said peptide that
        homes to said tumor is not present in said
20   normal tissue, thereby obtaining said tumor
        homing peptide,

        provided said tumor homing peptide is not an
antibody.
25

2. The conjugate of claim 1, wherein said
peptide contains the amino acid sequence RGD.

3. The conjugate of claim 1, wherein said
30 peptide contains the amino acid sequence NGR.

4. The conjugate of claim 1, wherein said
peptide contains the amino acid sequence GSL.

35   5. The conjugate of claim 1, wherein said
tumor homing peptide is NGRAHA (SEQ ID NO: 6) or CNGRC
(SEQ ID NO: 8).

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6.   The conjugate of claim 1, wherein·said tumor homing peptide is CDCRGDCFC (SEQ ID NO: 1)·.

7.   The conjugate of claim 1, wherein said tumor homing peptide is CNGRCVSGCAGRC (SEQ ID NO: 3) or CGSLVRC (SEQ ID NO: 5).

8.   The conjugate of claim 1, wherein said moiety is a cytotoxic agent.

9.   The conjugate of claim 1,·wherein said moiety is a drug..

10.   The conjugate of claim 9, wherein said drug is a cancer chemotherapeutic agent.

11.   The conjugate of claim 10, wherein said cancer chemotherapeutic agent is doxorubicin.

12.   The conjugate of claim 1, wherein said moiety is a detectable moiety.

13.   The conjugate of claim 1, wherein said moiety is selected from the group consisting of a chambered microdevice, a liposome, a cell and a virus.

14.   The conjugate of claim 1, wherein said moiety is a grafted polypeptide.

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15. A conjugate, comprising a tumor homing molecule linked to a moiety, said tumor homing molecule obtained by *in vivo* panning, comprising the steps of:

5

    a) administering to a first subject having a tumor a library of diverse molecules;

    b) collecting a sample of the tumor;

10

    c) identifying a molecule that homes to said tumor;

    d) collecting a sample of normal tissue corresponding to said tumor; and

15

    e) determining that said molecule that homes to said tumor is not present in said normal tissue, thereby obtaining said tumor homing molecule,

20

provided said tumor homing molecule is not an antibody.

16. The conjugate of claim 15, wherein said
25 molecule is a nucleic acid molecule.

17. The conjugate of claim 15, wherein said molecule is a peptidomimetic.

30   18. A conjugate, comprising a tumor homing peptide containing the amino acid sequence RGD, said tumor homing peptide linked to a moiety.

19. The conjugate of claim 18, wherein said
35 tumor homing peptide is CDCRGDCFC (SEQ ID NO: 1).

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20. A conjugate, comprising a tumor homing peptide containing the amino acid sequence NGR, said tumor homing peptide linked to a moiety.

5   21. The conjugate of claim 20, wherein said tumor homing peptide is NGRAHA (SEQ ID NO: 6).

22. The conjugate of claim 20, wherein said tumor homing peptide is CNGRC (SEQ ID NO: 8).

10

23. The conjugate of claim 20, wherein said tumor homing peptide is CNGRCVSGCAGRC (SEQ ID NO: 3).

24. A conjugate, comprising a tumor homing
15 peptide containing the amino acid sequence GSL, said tumor homing peptide linked to a moiety.

25. The conjugate of claim 24, wherein said tumor homing peptide is CGSLVRC (SEQ ID NO: 5).

20

26. A conjugate, comprising a tumor homing peptide selected from the group consisting of NGRAHA (SEQ ID NO: 6) and CNGRC (SEQ ID NO: 8), said tumor homing peptide linked to a moiety.

25

27. A conjugate, comprising CDCRGDCFC (SEQ ID NO: 1) linked to a moiety.

28. A conjugate, comprising a tumor homing
30 peptide selected from the group consisting of CNGRCVSGCAGRC (SEQ ID NO: 3) and CGSLVRC (SEQ ID NO: 5), said tumor homing peptide linked to a moiety.

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29. A tumor homing peptide identified by *in vivo* panning, comprising the steps of:

a) administering to a first subject
having a tumor a library of diverse peptides;

b) collecting a sample of the tumor;

c) identifying a peptide that homes to
said tumor;

d) collecting a sample of normal tissue
corresponding to said tumor; and

e) determining that said peptide that
homes to said tumor is not present in said
normal tissue, thereby identifying said peptide
as a tumor homing peptide,

provided said peptide is not an antibody.

30. The tumor homing peptide of claim 29,
wherein said sample of normal tissue corresponding to
said tumor is collected from said first subject.

31. The tumor homing peptide of claim 29,
wherein said sample of normal tissue corresponding to
said tumor is collected from a second subject.

32. The tumor homing peptide of claim 29,
wherein said tumor is a breast tumor.

33. The tumor homing peptide of claim 29,
wherein said tumor is a melanoma.

34. The tumor homing peptide of claim 29,
wherein said tumor is a Kaposi's sarcoma.

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35. A tumor homing peptide selected from the group consisting of CNGRCVSGCAGRC (SEQ ID NO: 3) and CGSLVRC (SEQ ID NO: 5).

36. A tumor homing molecule identified by *in vivo* panning, comprising the steps of:

a) administering to a first subject having a tumor a library of diverse molecules;

b) collecting a sample of the tumor;

c) identifying a molecule that homes to said tumor;

d) collecting a sample of normal tissue corresponding to said tumor; and

e) determining that said molecule that homes to said tumor is not present in said normal tissue, thereby identifying said molecule as a tumor homing molecule,

provided said molecule is not an antibody.

37. A method of directing a moiety to a tumor, comprising contacting the tumor with the conjugate of claim 1.

38. The method of claim 37, wherein said contacting step is performed *in vitro*.

39. The method of claim 37, wherein said contacting step is performed *in vivo*.

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40. A method of identifying the presence of a target molecule, which specifically binds a tumor homing molecule, wherein said tumor homing molecule is not an antibody, comprising contacting a sample of a tumor with

5   the tumor homing molecule and detecting specific binding of said tumor homing molecule to a component of said sample, said binding identifying the presence of a target molecule.

10   41. A method of identifying a target molecule, which is expressed in a tumor tissue, comprising the steps of:

           a) contacting a tumor tissue sample with

15   a tumor homing molecule that specifically binds to said tumor tissue, wherein said tumor homing molecule is not an antibody;

           b) identifying in said tumor tissue

20   sample target molecules bound by said tumor homing molecule;

           c) contacting a corresponding nontumor tissue sample with said tumor homing molecule;

25

           d) identifying in said corresponding nontumor tissue sample target molecules bound by said tumor homing molecule; and

30   e) comparing said target molecules of said tumor tissue with said target molecules of said corresponding nontumor tissue, thereby identifying a target molecule expressed by said tumor tissue, wherein said target molecule

35   specifically binds said tumor homing molecule.

●   ●

42. A method of obtaining a substantially isolated target molecule, which specifically binds a tumor homing molecule, comprising the step of substantially isolating the target molecule identified by

5   the method of claim 41.

43. A substantially isolated target molecule, which specifically binds a tumor homing molecule, obtained by the method of claim 42, provided said target

10   molecule is not an integrin.

44. A peptidomimetic, which competitively inhibits the binding of the target molecule of claim 43 to a naturally occurring ligand of the target molecule.

15

45. A molecule, which specifically binds to the target molecule of claim 43.

46. A molecule, which competitively inhibits

20   the binding of the target molecule of claim 43 to the tumor homing molecule.

47. The molecule of claim 46, which is a peptide.

25

48. The peptide of claim 47, which contains the amino acid sequence NGR.

49. The peptide of claim 48, which has the

30   amino acid sequence CNGRCVSGCAGRC (SEQ ID NO: 3).

50. The peptide of claim 47, which contains the amino acid sequence GSL.

35   51. The peptide of claim 50, which has the amino acid sequence CGSLVRC (SEQ ID NO: 5).

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52.   A target molecule, which is expressed in tumor vasculature, wherein said target molecule binds CNGRC (SEQ ID NO: 8) with a higher affinity than said target molecule binds CDCRGDCFC (SEQ ID NO: 1).

53.   An antibody that specifically binds the target molecule of claim 43.

54.   The antibody of claim 53, which is a monoclonal antibody.

55.   A method of directing a moiety in vivo to a tumor containing angiogenic vasculature, comprising contacting the tumor with the conjugate of claim 1.

56.   The method of claim 55, wherein said conjugate comprises a peptide containing the amino acid sequence RGD.

57.   The method of claim 55, wherein said conjugate comprises a peptide containing the amino acid sequence NGR.

58.   The method of claim 55, wherein said conjugate comprises a peptide containing the amino acid sequence GSL.

59.   The method of claim 55, wherein said conjugate comprises CDCRGDCFC (SEQ ID NO 1).

60.   The method of claim 55, wherein said conjugate comprises NGRAHA (SEQ ID NO: 6) or CNGRC (SEQ ID NO: 8).

61.   The method of claim 55, wherein said conjugate comprises CNGRCVSGCAGRC (SEQ ID NO: 3) or CGSLVRC (SEQ ID NO: 5).

62. The method of claim 55, wherein said conjugate comprises a moiety, which is a cytotoxic agent.

63. The method of claim 55, wherein said
5   conjugate comprises a moiety, which is a drug.

64. The method of claim 63, wherein said drug is a cancer chemotherapeutic agent.

10   65. The method of claim 64, wherein said cancer chemotherapeutic agent is doxorubicin.

66. The method of claim 55, wherein said conjugate comprises a moiety, which is a detectable
15   moiety.

67. The method of claim 55, wherein said conjugate comprises a moiety selected from the group consisting of a chambered microdevice, a liposome, a cell
20   and a virus.

68. The method of claim 55, wherein said conjugate comprises a moiety, which is a grafted polypeptide.
25

69. A method of targeting *in vivo* a tumor containing angiogenic vasculature, comprising contacting the tumor with a molecule that selectively binds an α_v-containing integrin.
30

70. The method of claim 69, said molecule selected from the group of an RGD-containing peptide and an antibody that selectively binds an α_v-containing integrin.
35

71. The method of claim 69, wherein said α_v-containing integrin is $\alpha_v\beta_3$ integrin.

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72. The method of claim 69, wherein said
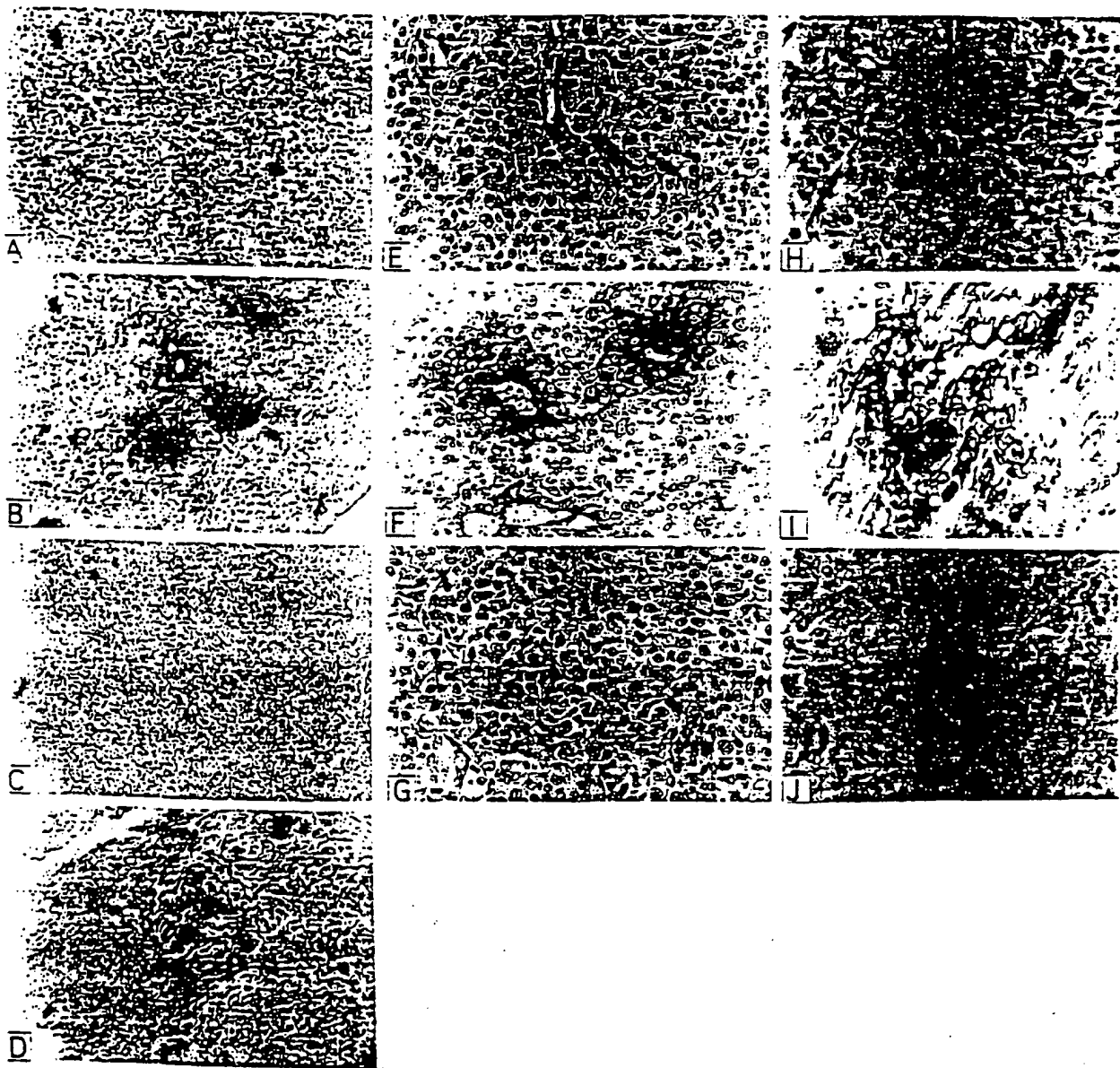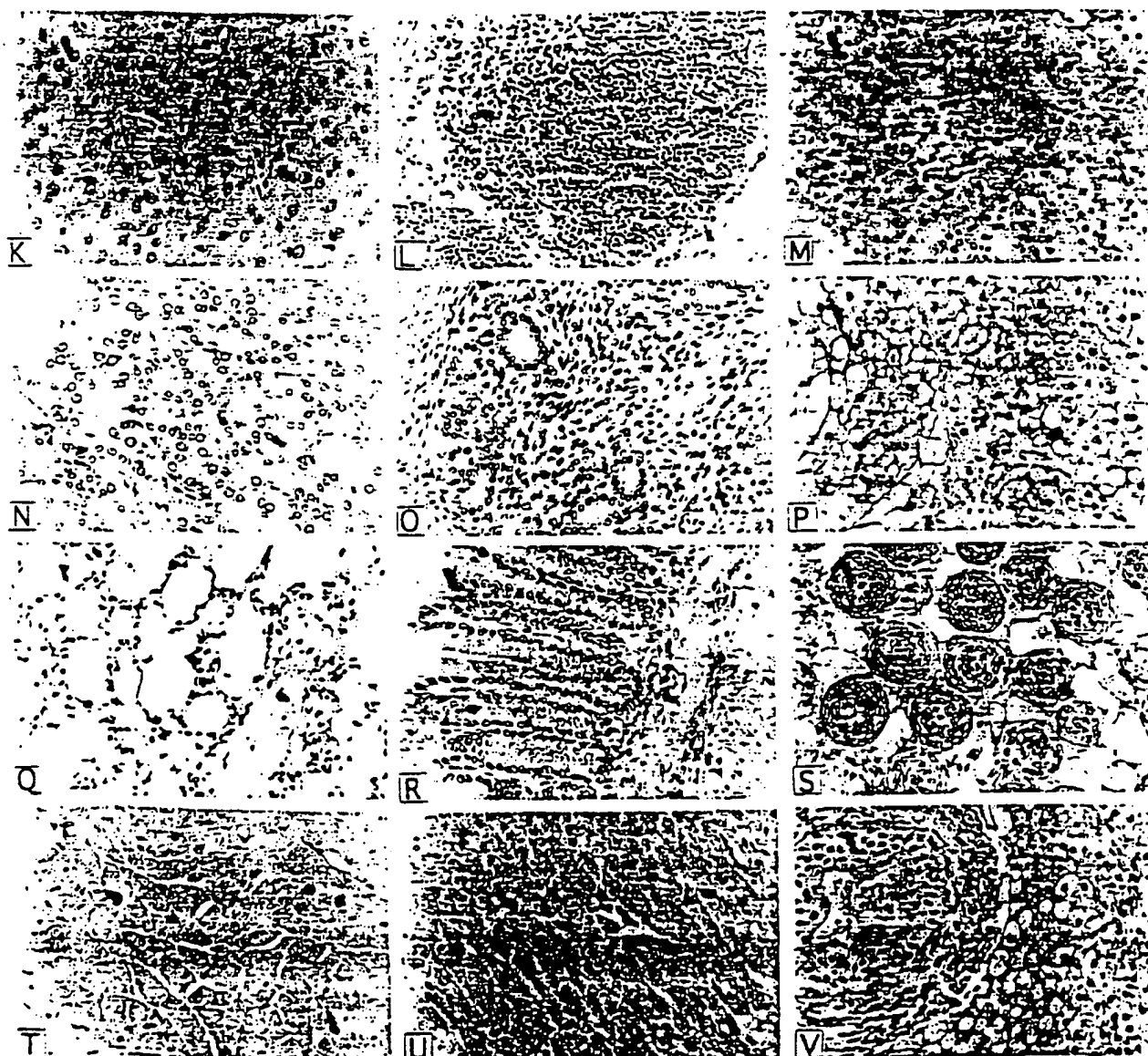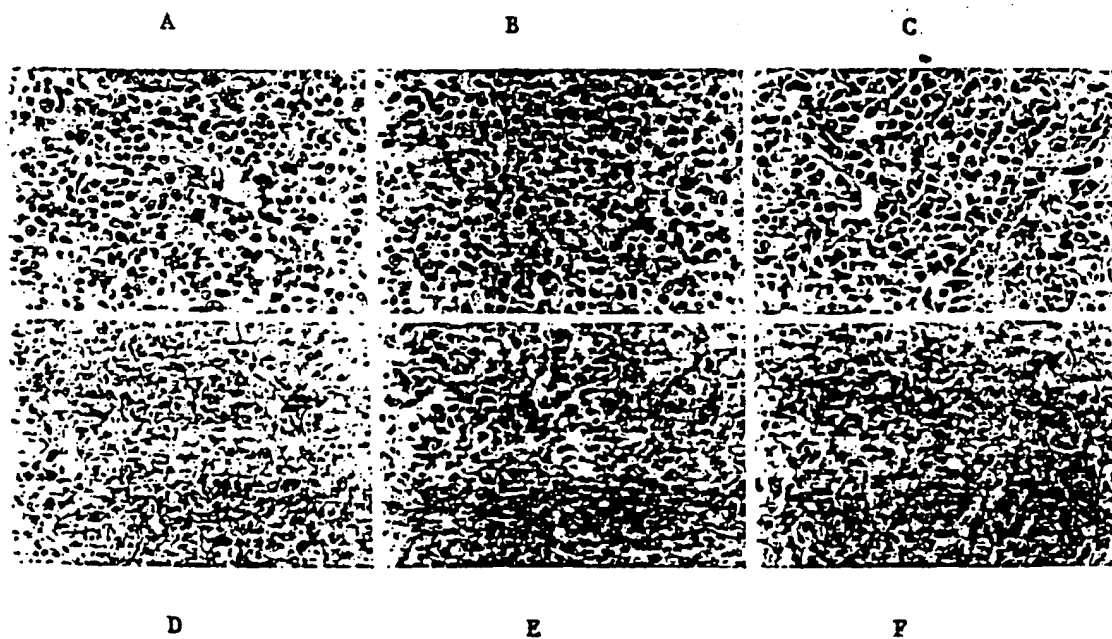molecule is CDCRGDCFC (SEQ ID NO: 14).

Figure 1

Figure 1 (cont.)

Figure 2